EXHIBIT A

NCBI HomoloGene Discover Homologs

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1: HomoloGene:55679. Gene conserved in Bilateria

Download, Links

Genes
Genes identified as putative homologs of one another during the construction of HomoloGene.

- H.sapiens PRKCA
  protein kinase C, alpha
- C.lupus LOC490904
  similar to Protein kinase C, alpha type (PKC-alpha) (PKC-A)
- M.musculus Prkca
  protein kinase C, alpha
- R.norvegicus Prkca
  protein kinase C, alpha
- G.gallus PRKCA
  protein kinase C, alpha
- D.melanogaster Pkc53E
  Protein C kinase 53E
- C.elegans pkc-2
  Protein Kinase C

Proteins
Proteins used in sequence comparisons and their conserved domain architectures.

- NP_002728.1
  672 aa
- XP_548026.2
  590 aa
- NP_035231.1
  672 aa
- XP_343976.3
  612 aa
- NP_001012822.1
  674 aa
- NP_725626.1
  679 aa
- NP_001024517.1
  682 aa

Alignment Scores
Various evolutionary parameters derived from pairwise alignments have been saved.

Conserved Domains
Conserved Domains from CDD found in protein sequences by rpsblast searching.

Show Table of Pairwise Scores

Alignments can be regenerated using BLAST for any selected pair of proteins.

Regenerate Alignments

NP_002728.1(H.sapiens. PRKCA)

XP_548026.2(C.lupus. LOC490904)

BLAST

Phenotypes

Phenotypic information for the genes in this entry imported from model organism databases.

D.melanogaster Pkc53E
Structural gene for protein kinase C (PKC) that is expressed primarily in the brain of adult flies.

H.sapiens MIM:176960
Pituitary tumor, invasive.

M.musculus MP:0002006
Tumorigenesis. Induction of new growth.

M.musculus MP:0002873
Normal phenotype.

M.musculus MP:0005369
Muscle phenotype.

M.musculus MP:0005385
Cardiovascular system phenotype.

M.musculus MP:0005393
Skin/coat/nails phenotype.

PubMed

Articles associated with genes and sequences of this entry plus additional related articles.

Stahelin RV, et al.
The origin of C1A-C2 interdomain interactions in protein kinase Calpha. J Biol Chem 280, 36452-36463 (2005).

Pascale A, et al.
Neuronal ELAV proteins enhance mRNA stability by a PKCalpha-

cd00180
S_TKc. Serine/Threonine protein kinases, catalytic domain
smart00133
S_TK_X. S_TK_X
cd00029
C1. Protein kinase C conserved region 1 (C1)
cd00276
C2_1. Protein kinase C conserved region 2, subgroup 1

Related Homology Resources

Links to curated and computed homology information found in other databases.

ZDB-GENE-040426-1178
Orthology group for D.rerio prkcb1 includes H.sapiens PRKCB1.

MGI:97595
Orthology group for M.musculus Prkca includes H.sapiens PRKCA and R.norvegicus Prkca.

UniGene

Links to groups of transcribed sequences established by tblastn searching of UniGene.

A.gambiae Aga.15734
Transcribed locus, weakly similar to XP_218062.4 similar to LATS homolog 1 ...

A.mellifera Ame.4093
Protein kinase C

B.mori Bmo.6810
Conventional protein kinase C

B.taurus Bt.1447
Protein kinase C, alpha

C.elegans Cel.667
Protein Kinase C

C.lupus Cfa.22766
Similar to Protein kinase C, alpha type (PKC-alpha) (PKC-A)

C.intestinalis Cin.1705
Transcribed locus, weakly similar to NP_001024516.1 Protein Kinase C family...

C.savignyi Csa.8810
Transcribed locus, weakly similar to NP_001027011.2 receptor kinase 7a [Dan...

dependent pathway. Proc Natl Acad Sci U S A 102, 12065-12070 (2005).

von Knethen A, et al.
Activation-induced depletion of protein kinase C alpha provokes desensitization of monocytes/macrophages in sepsis. J Immunol 174, 4960-4965 (2005).

Kirshenboim N, et al.
Lithium-mediated phosphorylation of glycogen synthase kinase-3beta involves PI3 kinase-dependent activation of protein kinase C-alpha. J Mol Neurosci 24, 237-245 (2004).

Hu T, et al.
Mechanisms of regulation of phospholipase D1 by protein kinase Calpha. J Biol Chem 278, 2348-2355 (2003).

Vichalkovski A, et al.
Tyrosine kinase modulation of protein kinase C activity regulates G protein-linked Ca2+ signaling in leukemic hematopoietic cells. Cell Calcium 39, 517-528 (2006).

Martelli AM, et al.
Molecular characterization of protein kinase C-alpha binding to lamin A. J Cell Biochem 86, 320-330 (2002).

Gu JL, et al.
Regulation of hematopoietic-specific G-protein Galpha15 and Galpha16 by protein kinase C. J Cell Biochem 88, 1101-1111 (2003).

Ranganathan S, et al.
Serine and threonine phosphorylation of the low density lipoprotein receptor-related protein by protein kinase Calpha regulates endocytosis and association with adaptor molecules. J Biol Chem 279, 40536-40544 (2004).

Ranganathan G, et al.
Regulation of lipoprotein lipase by protein kinase C alpha in 3T3-F442A adipocytes. J Biol Chem 277, 38669-38675 (2002).

Tigges U, et al.
The F-actin cross-linking and focal adhesion protein filamin A is a ligand and in vivo substrate for protein kinase C alpha. J Biol Chem 278, 23561-23569 (2003).

Jagadeesh S, et al.
Inositol hexaphosphate represses telomerase activity and translocates TERT from the nucleus in mouse and human prostate cancer cells via the deactivation of Akt and PKCalpha. Biochem Biophys Res Commun 349, 1361-1367 (2006).

Cardenas C, et al.
Depolarization of skeletal muscle cells induces phosphorylation of cAMP response element binding protein via calcium and protein kinase Calpha. J Biol Chem 279, 39122-39131 (2004).

Murakami M, et al.
Protein kinase C (PKC) delta regulates PKCalpha activity in a Syndecan-4-dependent manner. J Biol Chem 277, 20367-20371 (2002).

D.rerio Dr.33991
Im:7139045

D.rerio Dr.63380
Similar to Protein kinase C, beta 1, like

D.rerio Dr.84921
Protein kinase C, beta 1

D.rerio Dr.114481
Similar to protein kinase C, alpha type

D.rerio Dr.139271
Similar to Protein kinase C, beta 1, like

D.rerio Dr.139559
Similar to protein kinase C, alpha type

D.melanogaster Dm.3632
Protein C kinase 53E

D.melanogaster Dm.4688
Inactivation no afterpotential C

G.gallus Gga.7157
Protein kinase C beta 1

G.gallus Gga.39759
Protein kinase C, alpha

H.sapiens Hs.531704
Protein kinase C, alpha

M.fascicularis Mfa.1737
Transcribed locus, weakly similar to NP_001027011.2 receptor kinase 7a [Dan...

M.mulatta Mmu.5709
Protein kinase C, alpha

M.tectiformis Mte.2937
Transcribed locus, weakly similar to XP_363105.1 hypothetical protein MG086...

M.musculus Mm.222178
Protein kinase C, alpha

O.cuniculus Ocu.3268
Rabbit mRNA for protein kinase C gamma (PKC-gamma) (new proposed nomenclatur...

O.cuniculus Ocu.6469
Transcribed locus, weakly similar to NP_001027011.2 receptor kinase 7a [Dan...

O.aries Oar.11206
Transcribed locus, weakly similar to NP_001027011.2 receptor kinase 7a [Dan...

P.marinus Pma.6070
Protein kinase C

P.promelas Ppr.4345

Boskovic G, et al.
Regulation of retinoic acid receptor alpha by protein kinase C in B16 mouse melanoma cells. J Biol Chem 277, 26113-26119 (2002).

Maniar R, et al.
PKC alpha-dependent regulation of the IGF1 receptor in adult and embryonic rat cardiomyocytes. Mol Cell Biochem 275, 15-24 (2005).

Dykes AC, et al.
Microtubule-dependent PKC-alpha localization in A7r5 smooth muscle cells. Am J Physiol Cell Physiol 285, C76-87 (2003).

Klarl BA, et al.
Protein kinase C mediates erythrocyte "programmed cell death" following glucose depletion. Am J Physiol Cell Physiol 290, C244-253 (2006).

Alpini G, et al.
Ursodeoxycholate and tauroursodeoxycholate inhibit cholangiocyte growth and secretion of BDL rats through activation of PKC alpha. Hepatology 35, 1041-1052 (2002).

Lopez-Andreo MJ, et al.
Retinoic acid as a modulator of the activity of protein kinase Calpha. Biochemistry 44, 11353-11360 (2005).

Silva M, et al.
Salmonella typhimurium SipA-induced neutrophil transepithelial migration: involvement of a PKC-alpha-dependent signal transduction pathway. Am J Physiol Gastrointest Liver Physiol 286, G1024-1031 (2004).

Corbalan-Garcia S, et al.
A new phosphatidylinositol 4,5-bisphosphate-binding site located in the C2 domain of protein kinase Calpha. J Biol Chem 278, 4972-4980 (2003).

San-Juan-Vergara H, et al.
Protein kinase C-alpha activity is required for respiratory syncytial virus fusion to human bronchial epithelial cells. J Virol 78, 13717-13726 (2004).

Yao L, et al.
Evidence for a role of protein kinase C-alpha in urine concentration. Am J Physiol Renal Physiol 287, F299-304 (2004).

Hermoso M, et al.
Cell volume regulation in response to hypotonicity is impaired in HeLa cells expressing a protein kinase Calpha mutant lacking kinase activity. J Biol Chem 279, 17681-17689 (2004).

Transcribed locus, weakly similar to XP_342224.2 similar to Protein kinase ...

P.promelas Ppr.14140
Transcribed locus, weakly similar to NP_001027011.2 receptor kinase 7a [Dan...

P.promelas Ppr.15963
Transcribed locus, moderately similar to XP_343976.3 similar to Protein kin...

R.norvegicus Rn.86669
Protein kinase C, alpha

S.salar Ssa.26232
Transcribed locus, weakly similar to NP_001027011.2 receptor kinase 7a [Dan...

S.mansoni Sma.1220
Protein kinase C type beta

S.purpuratus Spu.15635
Similar to protein kinase C

S.purpuratus Spu.18631
Similar to Protein kinase C, alpha type (PKC-alpha) (PKC-A)

S.scrofa Ssc.15802
Protein kinase C, alpha

TaxID:9337 Tvu.7012
Transcribed locus, weakly similar to NP_001027011.2 receptor kinase 7a [Dan...

X.laevis Xl.34027
Transcribed locus, weakly similar to NP_006249.1 kinase, cGMP-dependent, ty...

X.laevis Xl.63817
Protein kinase C, alpha

X.laevis Xl.72710
Transcribed locus, weakly similar to XP_342224.2 similar to Protein kinase ...

X.tropicalis Str.35337
Hypothetical protein MGC146825

X.tropicalis Str.53046
CDNA clone IMAGE:7692520, containing frame-shift errors

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1: HomoloGene:55679. Gene conserved in Bilateria

Alignment Scores

Gene		Identity (%)		Substitution Rates [1]			
Species	Symbol	Protein	DNA	d	d_N/d_S	d_{NR}/d_{NC}	
H.sapiens	**PRKCA**						
vs. C.lupus	LOC490904	99.3	92.0	0.085	0.006	0.582	Blast
vs. M.musculus	Prkca	98.2	90.1	0.106	0.013	2.449	Blast
vs. R.norvegicus	Prkca	99.2	90.2	0.105	0.005	1.453	Blast
vs. G.gallus	PRKCA	94.5	83.3	0.189	0.018	0.478	Blast
vs. D.melanogaster	Pkc53E	70.8	66.9	0.437	undef	0.718	Blast
vs. C.elegans	pkc-2	69.0	63.9	0.492	undef	0.663	Blast
C.lupus	**LOC490904**						
vs. H.sapiens	PRKCA	99.3	92.0	0.085	0.006	0.582	Blast
vs. M.musculus	Prkca	97.9	88.1	0.129	0.011	0.776	Blast
vs. R.norvegicus	Prkca	99.7	89.5	0.113	0.003	0.289	Blast
vs. G.gallus	PRKCA	95.1	82.8	0.196	0.011	0.396	Blast
vs. D.melanogaster	Pkc53E	69.2	66.0	0.453	undef	0.697	Blast
vs. C.elegans	pkc-2	67.8	63.8	0.494	undef	0.737	Blast
M.musculus	**Prkca**						
vs. H.sapiens	PRKCA	98.2	90.1	0.106	0.013	2.449	Blast
vs. C.lupus	LOC490904	97.9	88.1	0.129	0.011	0.776	Blast
vs. R.norvegicus	Prkca	97.9	93.8	0.065	0.034	1.230	Blast

vs. G.gallus	PRKCA	93.0	82.7	0.197	0.021	0.562	Blast
vs. D.melanogaster	Pkc53E	70.1	66.6	0.443	undef	0.694	Blast
vs. C.elegans	pkc-2	68.1	63.8	0.494	undef	0.688	Blast
R.norvegicus	**Prkca**						
vs. H.sapiens	PRKCA	99.2	90.2	0.105	0.005	1.453	Blast
vs. C.lupus	LOC490904	99.7	89.5	0.113	0.003	0.289	Blast
vs. M.musculus	Prkca	97.9	93.8	0.065	0.034	1.230	Blast
vs. G.gallus	PRKCA	95.0	82.1	0.205	undef	0.436	Blast
vs. D.melanogaster	Pkc53E	69.1	66.1	0.451	undef	0.722	Blast
vs. C.elegans	pkc-2	68.1	64.5	0.481	undef	0.710	Blast
G.gallus	**PRKCA**						
vs. H.sapiens	PRKCA	94.5	83.3	0.189	0.018	0.478	Blast
vs. C.lupus	LOC490904	95.1	82.8	0.196	0.011	0.396	Blast
vs. M.musculus	Prkca	93.0	82.7	0.197	0.021	0.562	Blast
vs. R.norvegicus	Prkca	95.0	82.1	0.205	undef	0.436	Blast
vs. D.melanogaster	Pkc53E	70.9	65.9	0.455	undef	0.653	Blast
vs. C.elegans	pkc-2	71.0	64.2	0.487	undef	0.721	Blast
D.melanogaster	**Pkc53E**						
vs. H.sapiens	PRKCA	70.8	66.9	0.437	undef	0.718	Blast
vs. C.lupus	LOC490904	69.2	66.0	0.453	undef	0.697	Blast
vs. M.musculus	Prkca	70.1	66.6	0.443	undef	0.694	Blast
vs. R.norvegicus	Prkca	69.1	66.1	0.451	undef	0.722	Blast
vs. G.gallus	PRKCA	70.9	65.9	0.455	undef	0.653	Blast
vs. C.elegans	pkc-2	68.5	63.6	0.498	undef	0.839	Blast
C.elegans	**pkc-2**						
vs. H.sapiens	PRKCA	69.0	63.9	0.492	undef	0.663	Blast
vs. C.lupus	LOC490904	67.8	63.8	0.494	undef	0.737	Blast
vs. M.musculus	Prkca	68.1	63.8	0.494	undef	0.688	Blast
vs. R.norvegicus	Prkca	68.1	64.5	0.481	undef	0.710	Blast
vs. G.gallus	PRKCA	71.0	64.2	0.487	undef	0.721	Blast
vs. D.melanogaster	Pkc53E	68.5	63.6	0.498	undef	0.839	Blast

[1] We present three rates for nucleotide substitutions per site, as defined below:

- **d**: the number of nucleotide substitutions per site, corrected for multiple substitutions using the method of Jukes and Cantor (1969).
- **d_N/d_S**: the ratio of the rate of nonsynonymous substitutions (d_N) to the rate of synonymous substitutions(d_S), calculated using the method of Nei and Gojobori (1986). A high value of this metric indicates adaptive selection, whereas a low value indicates purifying selection.
- **d_{NR}/d_{NC}**: the ratio of radical nonsynonymous substitutions (d_{NR}) to conservative nonsynonymous substitutions (d_{NC}), calculated using the method of Hughes et al. (1990). This metric is analogous to d_N/d_S, but it has the advantage of being useful for studying the evolution of sequences that diverged in the distant past.

References

- Jukes TH, Cantor CR. Evolution of protein molecules. In: HN Munro, editor. Mammalian protein metabolism III. New York: Academic Press; 1969. p.21-132.
- Nei M, Gojobori T. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol Biol Evol. 1986;3(5):418-26.
- Hughes AL, Ota T, Nei M. Positive Darwinian selection promotes charge profile diversity in the antigen-binding cleft of class I MHC molecules. Mol Biol Evol. 1990;7(6):515-24.

EXHIBIT B

NCBI HomoloGene Discover Homologs... Help My NCBI [Sign In] [Register]

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Search HomoloGene for Go Clear

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All: 1 Fungi: 0 Mammals: 0

1: HomoloGene:56424. Gene conserved in Amniota

Download, Links

Genes
Genes identified as putative homologs of one another during the construction of HomoloGene.

- H.sapiens PRKCB1 protein kinase C, beta 1
- C.lupus LOC489968 similar to Protein kinase C, beta type (PKC-beta) (PKC-B)
- M musculus Prkcb1 protein kinase C, beta 1
- R norvegicus Prkcb1 protein kinase C, beta 1
- G.gallus LOC416567 protein kinase C, beta 1

Proteins
Proteins used in sequence comparisons and their conserved domain architectures.

- NP_002729.2 673 aa
- XP_547088.2 663 aa
- NP_032881.1 673 aa
- NP_036845.2 673 aa
- XP_414868.2 670 aa

Alignment Scores
Various evolutionary parameters derived from pairwise alignments have been saved.

Show Table of Pairwise Scores

Alignments can be regenerated using BLAST for any selected pair of proteins.

Conserved Domains
Conserved Domains from CDD found in protein sequences by rpsblast searching.

- cd00180
  - S_TKc. Serine/Threonine protein kinases, catalytic domain
- smart00133
  - S_TK_X. S_TK_X
- cd00029

Regenerate Alignments

NP_002729.2(H.sapiens. PRKCB1)

XP_547088.2(C.lupus. LOC489968)

BLAST

Phenotypes

Phenotypic information for the genes in this entry imported from model organism databases.

M.musculus MP:0003631
Nervous system phenotype.

M.musculus MP:0005386
Behavior/neurological phenotype.

M.musculus MP:0005387
Immune system phenotype.

M.musculus MP:0005397
Hematopoietic system phenotype.

PubMed

Articles associated with genes and sequences of this entry plus additional related articles.

Pinton P, et al.
Dynamics of glucose-induced membrane recruitment of protein kinase C beta II in living pancreatic islet beta-cells. J Biol Chem 277, 37702-37710 (2002).

Chen D, et al.
Centrosomal anchoring of protein kinase C betaII by pericentrin controls microtubule organization, spindle function, and cytokinesis. J Biol Chem 279, 4829-4839 (2004).

Liu Y, et al.
Protein kinase CbetaII regulates its own expression in rat intestinal epithelial cells and the colonic epithelium in vivo. J Biol Chem 279, 45556-45563 (2004).

Su TT, et al.
PKC-beta controls I kappa B kinase lipid raft recruitment and activation in response to BCR signaling. Nat Immunol 3, 780-786 (2002).

Li G, et al.

C1. Protein kinase C conserved region 1 (C1)
cd00276
C2_1. Protein kinase C conserved region 2, subgroup 1

Related Homology Resources

Links to curated and computed homology information found in other databases.

MGI:97596
Orthology group for M.musculus Prkcb1 includes H.sapiens PRKCB1 and R.norvegicus Prkcb1.

UniGene

Links to groups of transcribed sequences established by tblastn searching of UniGene.

B.taurus Bt.5435
Protein kinase C, beta 1

C.lupus Cfa.40232
Similar to Protein kinase C, beta type (PKC-beta) (PKC-B)

H.sapiens Hs.460355
Protein kinase C, beta 1

M.mulatta Mmu.1842
Protein kinase C, beta

M.musculus Mm.207496
Protein kinase C, beta 1

O.cuniculus Ocu.3267
Rabbit mRNA for protein kinase C alpha (PKC-alpha) (new proposed nomenclatur...

R.norvegicus Rn.91118
Protein kinase C, beta 1

S.scrofa Ssc.349
Protein kinase C beta 1

S.scrofa Ssc.4792
Protein kinase C, beta 1

Protein kinase C alpha, betaI, and betaII isozymes regulate cytokine production in mast cells through MEKK2/ERK5-dependent and -independent pathways. Cell Immunol 238, 10-18 (2005).

Saijo K, et al.
Protein kinase C beta controls nuclear factor kappaB activation in B cells through selective regulation of the IkappaB kinase alpha. J Exp Med 195, 1647-1652 (2002).

Suzuma K, et al.
Characterization of protein kinase C beta isoform's action on retinoblastoma protein phosphorylation, vascular endothelial growth factor-induced endothelial cell proliferation, and retinal neovascularization. Proc Natl Acad Sci U S A 99, 721-726 (2002).

Aleman G, et al.
Regulation by glucagon of the rat histidase gene promoter in cultured rat hepatocytes and human hepatoblastoma cells. Am J Physiol Endocrinol Metab 289, E172-179 (2005).

Birikh KR, et al.
Interaction of "readthrough" acetylcholinesterase with RACK1 and PKCbeta II correlates with intensified fear-induced conflict behavior. Proc Natl Acad Sci U S A 100, 283-288 (2003).

Helliwell PA, et al.
Intestinal sugar absorption is regulated by phosphorylation and turnover of protein kinase C betaII mediated by phosphatidylinositol 3-kinase- and mammalian target of rapamycin-dependent pathways. J Biol Chem 278, 28644-28650 (2003).

Philippi A, et al.
Haplotypes in the gene encoding protein kinase c-beta (PRKCB1) on chromosome 16 are associated with autism. Mol Psychiatry 10, 950-960 (2005).

Santiago-Walker AE, et al.
Protein kinase C delta stimulates apoptosis by initiating G1 phase cell cycle progression and S phase arrest. J Biol Chem 280, 32107-32114 (2005).

Zhang J, et al.
Protein kinase C (PKC) betaII induces cell invasion through a Ras/Mek-, PKC iota/Rac 1-dependent signaling pathway. J Biol Chem 279, 22118-22123 (2004).

Banan A, et al.
PKC-beta1 isoform activation is required for EGF-induced NF-kappaB inactivation and IkappaBalpha stabilization and protection of F-actin assembly and barrier function in enterocyte monolayers. Am J Physiol Cell Physiol 286, C723-738 (2004).

Yao LJ, et al.
Mice lacking protein kinase C beta present modest increases in systolic blood pressure and NH4Cl-induced metabolic acidosis. Kidney Blood Press Res 29, 36-42 (2006).

Broughman JR, et al.
Chronic PKC-beta activation in HT-29 Cl.19a colonocytes prevents cAMP-mediated ion secretion by inhibiting apical membrane current generation. Am J Physiol Gastrointest Liver Physiol 291, G318-330

(2006).

Lallemend F, et al.
Activation of protein kinase Cbetal constitutes a new neurotrophic pathway for deafferented spiral ganglion neurons. J Cell Sci 118, 4511-4525 (2005).

Liu Y, et al.
Critical role of protein kinase C betaII in activation of mast cells by monomeric IgE. J Biol Chem 280, 38976-38981 (2005).

Shcherbakov AM, et al.
Activated proteinkinase B in breast cancer. Bull Exp Biol Med 139, 608-610 (2005).

Araki S, et al.
Polymorphisms of the protein kinase C-beta gene (PRKCB1) accelerate kidney disease in type 2 diabetes without overt proteinuria. Diabetes Care 29, 864-868 (2006).

Venkataraman C, et al.
Selective role of PKCbeta enzymatic function in regulating cell survival mediated by B cell antigen receptor cross-linking. Immunol Lett 105, 83-89 (2006).

DelCarlo M, et al.
Chondrocyte cell death mediated by reactive oxygen species-dependent activation of PKC-betaI. Am J Physiol Cell Physiol 290, C802-811 (2006).

Bohlen HG
Protein kinase betaII in Zucker obese rats compromises oxygen and flow-mediated regulation of nitric oxide formation. Am J Physiol Heart Circ Physiol 286, H492-497 (2004).

Natsume Y, et al.
Dominant negative protein kinase Cbeta improves 1alpha, 25-dihydroxy vitamin D3-induced insulin resistance. Endocr Res 29, 457-464 (2003).

Banan A, et al.
The beta 1 isoform of protein kinase C mediates the protective effects of epidermal growth factor on the dynamic assembly of F-actin cytoskeleton and normalization of calcium homeostasis in human colonic cells. J Pharmacol Exp Ther 301, 852-866 (2002).

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Alignment Scores

Gene		Identity (%)		Substitution Rates [1]			
Species	Symbol	Protein	DNA	d	d_N/d_S	d_{NR}/d_{NC}	
H.sapiens	**PRKCB1**						
vs. C.lupus	LOC489968	94.7	89.6	0.112	0.069	0.686	Blast
vs. M.musculus	Prkcb1	98.8	91.5	0.090	0.009	0.198	Blast
vs. R.norvegicus	Prkcb1	98.7	91.4	0.092	0.011	0.240	Blast
vs. G.gallus	LOC416567	90.2	80.9	0.220	0.036	0.783	Blast
C.lupus	**LOC489968**						
vs. H.sapiens	PRKCB1	94.7	89.6	0.112	0.069	0.686	Blast
vs. M.musculus	Prkcb1	95.3	87.6	0.135	0.042	0.881	Blast
vs. R.norvegicus	Prkcb1	95.2	87.7	0.134	0.044	0.805	Blast
vs. G.gallus	LOC416567	94.6	83.4	0.187	0.021	0.554	Blast
M.musculus	**Prkcb1**						
vs. H.sapiens	PRKCB1	98.8	91.5	0.090	0.009	0.198	Blast
vs. C.lupus	LOC489968	95.3	87.6	0.135	0.042	0.881	Blast
vs. R.norvegicus	Prkcb1	99.9	96.6	0.034	0.004	0	Blast
vs. G.gallus	LOC416567	90.9	81.0	0.219	0.034	0.863	Blast
R.norvegicus	**Prkcb1**						
vs. H.sapiens	PRKCB1	98.7	91.4	0.092	0.011	0.240	Blast
vs. C.lupus	LOC489968	95.2	87.7	0.134	0.044	0.805	Blast
vs. M.musculus	Prkcb1	99.9	96.6	0.034	0.004	0	Blast

vs. G.gallus	LOC416567	90.7	80.6	0.225	0.032	0.877	Blast
G.gallus	**LOC416567**						
vs. H.sapiens	PRKCB1	90.2	80.9	0.220	0.036	0.783	Blast
vs. C.lupus	LOC489968	94.6	83.4	0.187	0.021	0.554	Blast
vs. M.musculus	Prkcb1	90.9	81.0	0.219	0.034	0.863	Blast
vs. R.norvegicus	Prkcb1	90.7	80.6	0.225	0.032	0.877	Blast

[1] We present three rates for nucleotide substitutions per site, as defined below:

- **d**: the number of nucleotide substitutions per site, corrected for multiple substitutions using the method of Jukes and Cantor (1969).
- $\mathbf{d_N/d_S}$: the ratio of the rate of nonsynonymous substitutions (d_N) to the rate of synonymous substitutions(d_S), calculated using the method of Nei and Gojobori (1986). A high value of this metric indicates adaptive selection, whereas a low value indicates purifying selection.
- $\mathbf{d_{NR}/d_{NC}}$: the ratio of radical nonsynonymous substitutions (d_{NR}) to conservative nonsynonymous substitutions (d_{NC}), calculated using the method of Hughes et al. (1990). This metric is analogous to d_N/d_S, but it has the advantage of being useful for studying the evolution of sequences that diverged in the distant past.

References

- Jukes TH, Cantor CR. Evolution of protein molecules. In: HN Munro, editor. Mammalian protein metabolism III. New York: Academic Press; 1969. p.21-132.
- Nei M, Gojobori T. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol Biol Evol. 1986;3(5):418-26.
- Hughes AL, Ota T, Nei M. Positive Darwinian selection promotes charge profile diversity in the antigen-binding cleft of class I MHC molecules. Mol Biol Evol. 1990;7(6):515-24.

EXHIBIT C

NCBI HomoloGene Discover Homologs

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Display HomoloGene Show 20 Send to

All: 1 Fungi: 0 Mammals: 1

1: HomoloGene:20602. Gene conserved in Eutheria

Download, Links

Genes
Genes identified as putative homologs of one another during the construction of HomoloGene.

H.sapiens PRKCG
protein kinase C, gamma

C.lupus LOC484316
similar to Protein kinase C, gamma type (PKC-gamma)

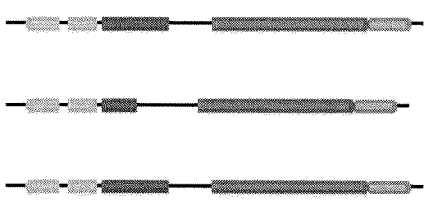
R.norvegicus Prkcc
protein kinase C, gamma

Proteins
Proteins used in sequence comparisons and their conserved domain architectures.

NP_002730.1
697 aa

XP_541432.2
673 aa

NP_036760.1
697 aa



Alignment Scores
Various evolutionary parameters derived from pairwise alignments have been saved.

Show Table of Pairwise Scores

Alignments can be regenerated using BLAST for any selected pair of proteins.

Regenerate Alignments

NP_002730.1(H.sapiens. PRKCG)
XP_541432.2(C.lupus. LOC484316)

Conserved Domains
Conserved Domains from CDD found in protein sequences by rpsblast searching.

cd00180
S_TKc. Serine/Threonine protein kinases, catalytic domain
smart00133
S_TK_X. S_TK_X
cd00029
C1. Protein kinase C conserved region 1 (C1)
cd00276
C2_1. Protein kinase C conserved region 2, subgroup 1

BLAST

Phenotypes

Phenotypic information for the genes in this entry imported from model organism databases.

H.sapiens MIM:605361
Spinocerebellar ataxia 14.

PubMed

Articles associated with genes and sequences of this entry plus additional related articles.

Rodriguez I, et al.
Activation of phospholipase C-gamma1 in human keratinocytes by hyperosmolar shock without enzyme phosphorylation. Arch Dermatol Res 295, 490-497 (2004).

Boyle AJ, et al.
Inhibition of protein kinase C reduces left ventricular fibrosis and dysfunction following myocardial infarction. J Mol Cell Cardiol 39, 213-221 (2005).

Seki T, et al.
Mutant protein kinase Cgamma found in spinocerebellar ataxia type 14 is susceptible to aggregation and causes cell death. J Biol Chem 280, 29096-29106 (2005).

Hung AC, et al.
Roles of protein kinase C in regulation of P2X7 receptor-mediated calcium signalling of cultured type-2 astrocyte cell line, RBA-2. Cell Signal 17, 1384-1396 (2005).

Hamplova B, et al.
Protein kinase C activity and isoform expression during early postnatal development of rat myocardium. Cell Biochem Biophys 43, 105-117 (2005).

Klebe S, et al.
New mutations in protein kinase Cgamma associated with spinocerebellar ataxia type 14. Ann Neurol 58, 720-729 (2005).

van de Warrenburg BP, et al.
Identification of a novel SCA14 mutation in a Dutch autosomal dominant cerebellar ataxia family. Neurology 61, 1760-1765 (2003).

Al-Fayez M, et al.
Deficits in the mid-brain raphe nuclei and striatum of the AS/AGU rat, a protein kinase C-gamma mutant. Eur J Neurosci 22, 2792-2798

UniGene

Links to groups of transcribed sequences established by tblastn searching of UniGene.

B.taurus Bt.12762
Protein kinase C, gamma

D.rerio Dr.88228
Protein kinase C, beta 1, like

H.sapiens Hs.631564
Protein kinase C, gamma

H.magnipapillata Hma.10090
Transcribed locus

M.fascicularis Mfa.450
Macaca fascicularis brain cDNA, clone: QccE-17190, similar to human protein ...

M.fascicularis Mfa.8657
Transcribed locus, weakly similar to XP_343976.3 similar to Protein kinase ...

M.fascicularis Mfa.9033
Transcribed locus, weakly similar to NP_001027011.2 receptor kinase 7a [Dan...

M.mulatta Mmu.15377
Similar to protein kinase C, gamma

M.musculus Mm.7980
Protein kinase C, gamma

M.musculus Mm.436654
Protein kinase C, gamma

O.mykiss Omy.20209
Transcribed locus, moderately similar to XP_343976.3 similar to Protein kin...

O.cuniculus Ocu.1950
Protein kinase C, gamma

R.norvegicus Rn.9747
Protein kinase C, gamma

X.laevis Xl.69670
Transcribed locus, weakly similar to XP_001058582.1 similar to Ribosomal pr...

X.tropicalis Str.59334
Transcribed locus

X.tropicalis Str.60831
Transcribed locus

(2005).

Codazzi F, et al.
Synergistic control of protein kinase Cgamma activity by ionotropic and metabotropic glutamate receptor inputs in hippocampal neurons. J Neurosci 26, 3404-3411 (2006).

Yabe I, et al.
Spinocerebellar ataxia type 14 caused by a mutation in protein kinase C gamma. Arch Neurol 60, 1749-1751 (2003).

Chen DH, et al.
Missense mutations in the regulatory domain of PKC gamma: a new mechanism for dominant nonepisodic cerebellar ataxia. Am J Hum Genet 72, 839-849 (2003).

Mochizuki H, et al.
R659S mutation of gammaPKC is susceptible to cell death: implication of this mutation/polymorphism in the pathogenesis of retinitis pigmentosa. Neurochem Int 49, 669-675 (2006).

Colombo PJ, et al.
Individual differences in spatial memory among aged rats are related to hippocampal PKCgamma immunoreactivity. Hippocampus 12, 285-289 (2002).

Rosenberg M, et al.
Protein kinase Cgamma regulates myosin IIB phosphorylation, cellular localization, and filament assembly. Mol Biol Cell 17, 1364-1374 (2006).

Nakamura J
Protein kinase C attenuates beta-adrenergic receptor-mediated lipolysis, probably through inhibition of the beta1-adrenergic receptor system. Arch Biochem Biophys 447, 1-10 (2006).

Matsumoto S, et al.
Protein kinase C-gamma and calcium/calmodulin-dependent protein kinase II-alpha are persistently translocated to cell membranes of the rat brain during and after middle cerebral artery occlusion. J Cereb Blood Flow Metab 24, 54-61 (2004).

Shumilla JA, et al.
Ethanol withdrawal-associated allodynia and hyperalgesia: age-dependent regulation by protein kinase C epsilon and gamma isoenzymes. J Pain 6, 535-549 (2005).

Ananthanarayanan B, et al.
Activation mechanisms of conventional protein kinase C isoforms are determined by the ligand affinity and conformational flexibility of their C1 domains. J Biol Chem 278, 46886-46894 (2003).

Xu GG, et al.
Prolonged Alzheimer-like tau hyperphosphorylation induced by simultaneous inhibition of phosphoinositol-3 kinase and protein kinase C in N2a cells. Acta Biochim Biophys Sin (Shanghai) 37, 349-354 (2005).

Uchino M, et al.
Isoform-specific phosphorylation of metabotropic glutamate receptor 5 by protein kinase C (PKC) blocks Ca2+ oscillation and oscillatory

1: HomoloGene:20602. Gene conserved in Eutheria

Alignment Scores

Gene		Identity (%)		Substitution Rates [1]			
Species	Symbol	Protein	DNA	d	d_N/d_S	d_{NR}/d_{NC}	
H.sapiens	**PRKCG**						
vs. C.lupus	LOC484316	94.5	89.0	0.119	0.085	0.956	Blast
vs. R.norvegicus	Prkcc	99.0	90.0	0.108	0.009	0.635	Blast
C.lupus	**LOC484316**						
vs. H.sapiens	PRKCG	94.5	89.0	0.119	0.085	0.956	Blast
vs. R.norvegicus	Prkcc	94.3	85.3	0.164	0.052	0.939	Blast
R.norvegicus	**Prkcc**						
vs. H.sapiens	PRKCG	99.0	90.0	0.108	0.009	0.635	Blast
vs. C.lupus	LOC484316	94.3	85.3	0.164	0.052	0.939	Blast

[1] We present three rates for nucleotide substitutions per site, as defined below:

- **d**: the number of nucleotide substitutions per site, corrected for multiple substitutions using the method of Jukes and Cantor (1969).
- **d_N/d_S**: the ratio of the rate of nonsynonymous substitutions (d_N) to the rate of synonymous substitutions(d_S), calculated using the method of Nei and Gojobori (1986). A high value of this metric indicates adaptive selection, whereas a low value indicates purifying selection.

- **d_{NR}/d_{NC}**: the ratio of radical nonsynonymous substitutions (d_{NR}) to conservative nonsynonymous substitutions (d_{NC}), calculated using the method of Hughes et al. (1990). This metric is analogous to d_N/d_S, but it has the advantage of being useful for studying the evolution of sequences that diverged in the distant past.

References

- Jukes TH, Cantor CR. Evolution of protein molecules. In: HN Munro, editor. Mammalian protein metabolism III. New York: Academic Press; 1969. p.21-132.
- Nei M, Gojobori T. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol Biol Evol. 1986;3(5):418-26.
- Hughes AL, Ota T, Nei M. Positive Darwinian selection promotes charge profile diversity in the antigen-binding cleft of class I MHC molecules. Mol Biol Evol. 1990;7(6):515-24.

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RNA Interference in Biology and Medicine

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***Abstract*——First discovered in plants the nematode *Caenorhabditis elegans*, the production of small interfering RNAs (siRNAs) that bind to and induce the degradation of specific endogenous mRNAs is now recognized as a mechanism that is widely employed by eukaryotic cells to inhibit protein production at a post-transcriptional level. The endogenous siRNAs are typically 19- to 23-base double-stranded RNA oligonucleotides, produced from much larger RNAs that upon binding to target mRNAs recruit RNases to a protein complexthat degrades the targeted mRNA. Methods for expressing siRNAs in cells in culture and in vivo using viral vectors, and for transfecting cells with synthetic siRNAs, have been developed and are being used to establish the functions of specific proteins in various cell types and organisms. RNA interference methods provide several major advantages over prior methods (antisense DNA or antibody-based techniques) for suppressing gene expression. Recent preclinical studies suggest that RNA interference technology holds promise for the treatment of various diseases. Pharmacologists have long dreamed of the ability to selectively antagonize or eliminate the function of individual proteins—RNAi technology may eventually make that dream a reality.**

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I. Introduction

RNA interference (RNAi[1]), as commonly defined, is a phenomenon leading to post-transcriptional gene silencing (PTGS) after endogenous production or artificial introduction into a cell of small interfering double strand RNA (siRNA) with sequences complementary to the targeted gene (Bosher and Labouesse, 2000). Whereas the transcription of the gene is normal, the translation of the protein is prevented by selective degradation of its encoded mRNA. However, PTGS is not restricted to RNAi and has emerged as a more complex mechanism that involves several different proteins and small RNAs. It is presumed that cells employ RNAi to tightly regulate protein levels in response to various environmental stimuli, although the extent to which this mechanism is employed by specific cell types remains to be discovered. However, the fact that RNAi is operative in cells of organisms ranging from plants, to nematodes and flies, and to mammals attests to its fundamental importance in the selective suppression of protein translation by targeted degradation of the encoding mRNA. Beyond its biological relevance, PTGS is emerging as a powerful tool to study the function of individual proteins or sets of proteins. User-friendly technologies for introducing siRNA into cells, in culture or in vivo, to achieve a selective reduction of single or multiple proteins of interest are rapidly evolving. The present article reviews this emerging technology, findings obtained to date using such RNAi methods, and the potential of RNAi-based therapeutics for treating human disease.

II. Principles of RNA Interference

RNA interference most likely evolved as a mechanism for cells to eliminate unwanted foreign genes. Foreign genes are often present in cells at high copy numbers, being present as viral genes, transposable elements, or as plasmids introduced experimentally in cell transfection protocols. It has been known for several decades that the level of expression of transgenes usually decreases as the number of copies present in the cell increases and that endogenous homologous genes can also be suppressed by the presence of the transgene (Napoli et al., 1990). Although such gene silencing can occur at the transcriptional level, it is now recognized that a major mechanism of gene suppression occurs post-transcriptionally, and that a major mechanism for this PTGS is RNAi, the selective degradation of mRNAs targeted by siRNAs (Van Blokland et al., 1994). Such PTGS via RNAi can occur very rapidly with proteins for many genes, being decreased within hours, and completely absent within 24 h (Pruss et al., 1997). Based upon these and other findings initially made in studies of plants (Ratcliff et al., 1997), it seems very likely that RNAi evolved as a mechanism to defend plant cells against viral infections.

A. Post-Transcriptional Gene Silencing and the Discovery of RNA Interference

PTGS and RNAi were discovered in genetic transformation studies of eukaryotic cells, principally plants and worms, wherein it was shown that mRNAs for the encoded transgene alone, or together with mRNAs for homologous endogenous genes are very low or absent despite high levels of transcription (Fire, 1999; Marathe et al., 2000). The ability to manipulate and monitor gene expression in the plant *Arabidopsis thaliana* and the roundworm *Caenorhabditis elegans* (the genomes of both species are now complete) revealed the process of RNAi and allowed the relatively rapid identification of several genes that regulate the RNAi process.

Transgenes insert into the genomes of plants by recombination in an apparently random manner so that the number of inserted copies, their chromosomal location, and their local arrangement within the chromosome vary among transformants. The observation of an inverse correlation between copy number and the level of gene expression suggested that an increased copy number of a particular gene results in silencing of that gene (Assaad et al., 1993). It was initially thought that such gene silencing was due to reduced gene transcription resulting from interactions between closely linked copies that result in the formation of secondary structures that promote methylation and inhibition of transcription (Ye and Signer, 1996). Further studies showed that transcriptional gene silencing (TGS) could also occur in trans, such that one transgene can be silenced by another transgene introduced either by crossing or transformation. It was then proposed that a silencing RNA is produced by one locus that somehow effects the silencing of the other gene by a mechanism involving RNA-mediated inhibition of transcription (Mette et al., 2000). Although some data were consistent with such mechanisms of transcriptional silencing, additional data suggested the involvement of PTGS. The presence of double-stranded RNAs (dsRNAs) and their cleavage into siRNAs of approximately 23 nucleotides was demonstrated, and it was then shown that expression of dsRNA with sequences corresponding to open reading frames in plants results in PTGS (Hamilton and Baulcombe, 1999). Similarly, expression of dsRNAs with sequences complementary to those of endogenous genes

[1]Abbreviations: RNAi, RNA interference; AIF, apotosis-inducing factor; dsRNA, double-stranded RNA; IAP, inhibitor of apoptosis protein; PTGS, post-transcriptional gene silencing; RISC, RNA-induced silencing complex; shRNA, short hairpin RNA; siRNA, small interfering RNA; IP_3, inositol 1,4,5-trisphosphate; NMD, nonsense-mediated mRNA decay; miRNA, micro-RNA; GFP, green fluorescent protein; NF-κB, nuclear factor-κB; HIV, human immunodeficiency virus; TNF, tumor necrosis factor; TGS, transcriptional gene silencing.

results in the selective silencing of those genes in *C. elegans* (Zamore et al., 2000). Collectively, the studies of *A. thaliana* and *C. elegans* showed that both TGS and PTGS can be initiated by the same RNA degradation pathway—TGS occurs when the dsRNA includes promoter sequences, whereas PTGS occurs when the dsRNA includes coding sequences. Although degradation of dsRNA is common to both mechanisms of gene silencing, the results also indicated that dsRNA-mediated TGS and PTGS involve different specific steps.

Although RNAi as a mechanism of PTGS was first discovered in plants and may have evolved as a cellular defense mechanism against foreign DNA and RNA, it is very clear that RNAi is widely employed in most if not all eukaryotic cells as a mechanism to regulate the expression of endogenous genes. In 1998, it was discovered that injection of dsRNA was much more effective for silencing of gene expression in *C. elegans* than was single-stranded antisense RNA (Fire et al., 1998). This experimentally induced PTGS, the first report of the use of RNAi as a tool in biology, was very potent, and remarkably, the PTGS occurred not only in the worms to which the dsRNA was administered, but also in their progeny. It was then demonstrated that the endogenous mRNA was the target of the injected dsRNA by a post-transcriptional mechanism and involving degradation of the targeted mRNA (Montgomery et al., 1998). Surprisingly, it was further shown that the dsRNA is effective at very low concentrations, such that the copy numbers of the targeted mRNA are far greater than the number of dsRNAs present in the cell (Fire et al., 1998; Kennerdell and Carthew, 1998). In addition, the suppression of the protein encoded by the targeted mRNA was found to persist through many rounds of cell division. The latter two observations strongly suggested that cells possess a mechanism for amplifying the RNAi mechanism. Not only can the RNAi process be maintained within cells of a common lineage, but it can also be transferred between cells, as shown in *C. elegans* where injection of dsRNA into the intestine results in silencing of the targeted gene in all cells of the F1 progeny of that worm (Fire et al., 1998). Indeed, dsRNA can enter cells and induce PTGS when worms are soaked in a solution containing the dsRNA or when the worms are fed bacteria expressing dsRNA (Tabara et al., 1998; Timmons and Fire, 1998). Recently, a transmembrane protein called SID-1 was identified as a possible mediator of intercellular transfer of RNAi (Winston et al., 2002).

Subsequently, other organisms were assayed for their capacity to induce RNAi. Evidence for RNAi in Drosophila was first demonstrated by Kennerdell and coworkers (Kennerdell and Carthew, 1998) who showed the involvement of the *frizzled* and *frizzled2* genes in the wingless pathway after introduction of dsRNA into embryos. Again, several techniques were developed in order to use dsRNA in this organism leading to the establishment of cell-free (Tuschl et al., 1999) and cell culture models (Caplen et al., 2000). A system that employed dsRNA as an extended hairpin-loop RNA was developed to induce heritable gene silencing (Kennerdell and Carthew, 2000). The *Drosophila* system has allowed the identification of several endogenous genes that play key roles in the RNAi process. An RNA nuclease activity called RISC (RNA-induced silencing complex) was discovered that is responsible for the degradation of endogenous mRNAs, as well as small nucleotide fragments (~25 nucleotides in length), which could be used as guides by RISC (Hammond et al., 2000). They later characterized RISC as a ribonucleoproteic complex (Hammond et al., 2001). These results were soon extended by showing that RNAi is an ATP-dependent and translation-independent event where the introduced dsRNA is processed into 21–23 nucleotide fragments that guide the cleavage of endogenous transcripts (Zamore et al., 2000). The enzyme responsible for the processing of the dsRNA was later discovered as a RNase III family nuclease named Dicer, a protein with high homology to the *rde-1 C. elegans* gene (Bernstein et al., 2001). To study the functions of RNAi in yeast, Volpe et al. (2002) deleted argonaute, dicer, and RNA-dependent RNA polymerase homologs; deletion resulted in the accumulation of complementary transcripts from centromeric heterochromatic repeats and de-repression of transgenes integrated at the centromere and impairment of centromere function. The authors of the latter study proposed that dsRNA arising from centromeric repeats targets the formation and maintenance of heterochromatin through RNAi.

In mammalian cells, RNAi was first employed as a tool to induce the silencing of the targeted gene (Wianny and Zernicka-Goetz, 2000). This approach was partially successful in mouse embryos (Wianny and Zernicka-Goetz, 2000; Svoboda et al., 2001) and embryonic cell lines (Billy et al., 2001; Yang et al., 2001; Paddison et al., 2002b) where specific gene silencing was achieved. On the other hand, the introduction of dsRNA into mammalian somatic cells presents a major problem because it can induce (in a manner similar to the silencing observed during viral infection) to the activation of the PKR (protein kinase R) and RNAseL pathway, resulting in the inhibition of protein synthesis and induction of apoptosis (Baglioni and Nilsen, 1983; Clarke and Mathews, 1995; Gil and Esteban, 2000). Interestingly, this shows that, in mammalian cells, the mechanisms for RNA interference are not identical to those in lower organisms although RNAi does operate in at least a subset of mammalian cell types, in a Dicer-dependent manner via post-transcriptional mechanisms (Billy et al., 2001; Paddison et al., 2002b). Elbashir et al. (2001a) had the idea of directly introducing 21–23 nucleotide dsRNA (siRNAs) into mouse and human cells to try to avoid the problems associated with the expression of longer dsRNAs. They showed that the siRNA could efficiently trigger silencing in the mammalian cells.

RNAi mechanisms may provide explanations for various biological phenomena that were previously described, but without any understanding of the possible underlying mechanism. For example, it was recently proposed that RNAi mechanisms could explain the controversial process of RNA-mediated memory transfer in planaria (Smalheiser et al., 2001). It will certainly be of interest to investigate possible roles for RNAi in the many different physiological processes that involve modulation of protein levels at a post-transcriptional level.

B. Mechanism of RNA Interference

A clearer picture of PTGS emerged from several different basic observations, including the necessity of transcriptionally active genes and the ability of RNA viruses to silence a homologous endogenous gene (English et al., 1997). Within the last 3 years, a flurry of studies have identified several of the molecules that mediate RNAi, and the mechanism whereby these molecules effect the selective degradation of targeted mRNAs. It is now clear that the production of dsRNA with sequence complementary to the mRNA being targeted is fundamental to the process of PTGS; single-stranded RNA is not sufficient to induce PTGS. The importance of dsRNA is supported by a wealth of data. Transgenes engineered to synthesize dsRNA require only a few copies of the dsRNA to achieve PTGS and can induce cosuppression. There are several ways such transgenes produce dsRNA including the synthesis of long hairpin mRNAs by transcription of an inverted repeat (Kennerdell and Carthew, 2000; Tavernarakis et al., 2000), and transcription of complementary sense and antisense strands by opposing promoters (Wang et al., 2000). Other studies have shown that although cells may initially produce very long dsRNAs, they are cleaved into smaller dsRNAs, 21–25 nucleotides in length, that actually mediate RNAi (Hamilton and Baulcombe, 1999).

How does a small dsRNA with a sequence complementary to a specific mRNA effect PTGS? The proteins that mediate the RNAi process have been identified using several approaches, most notably genetic screens for mutants resistant to RNAi in *C. elegans* and resistant to PTGS in *Neurospora* and *Arabidopsis* (Fig. 2). These studies have identified homologous genes in each species, and subsequent identification of mammalian homologs, that encode proteins required for RNAi. Three homologous genes identified in the initial screens are *rde-1* in *C. elegans* (Tabara et al., 1999), *qde-2* in *Neurospora* (Cogoni et al., 1996) and *ago-1* in *Arabidopsis* (Dalmay et al., 2000; Fagard et al., 2000). The proteins encoded by each of these genes share homologies with the eIF2C translation factors. In *C. elegans*, *rde-1* is required for germline transmission of RNAi, but not for transmission of RNAi among cells of the worm (Grishok

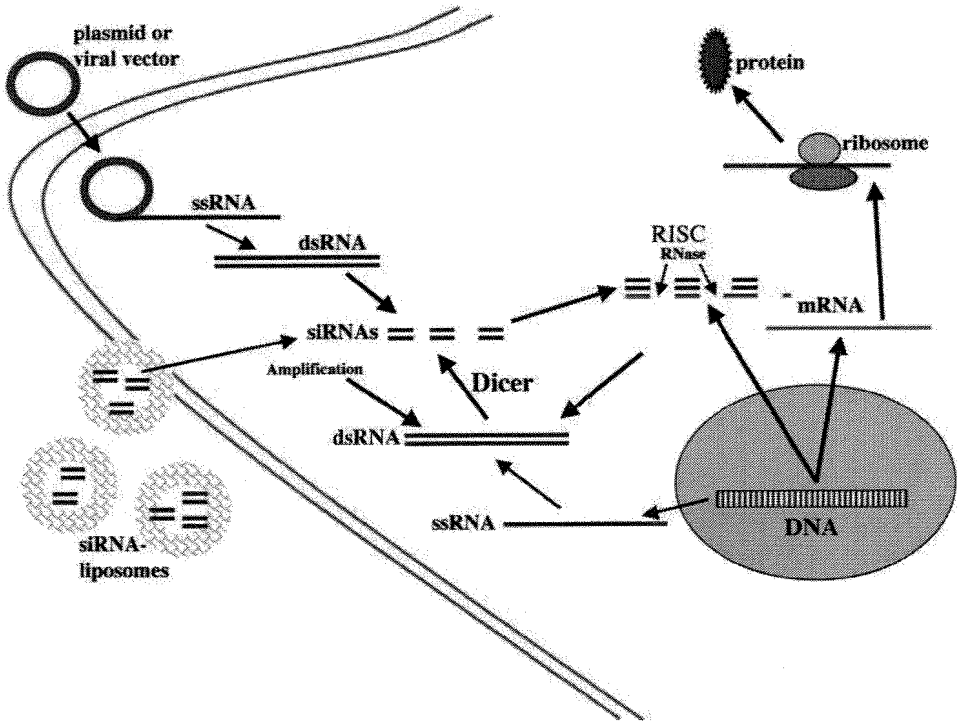

FIG. 1. Pathways of RNAi. Cells produce single-stranded RNA (ssRNA), which provide a template for the formation of dsRNA, which involves the activity of RNA-dependent RNA polymerases. The dsRNA is then cleaved by a protein called Dicer to form small 21–23 nucleotide siRNAs. The siRNAs (blue) then associate with the specific mRNA targeted by their nucleotide sequence (red) in a nucleic acid-protein complex called RISC, which includes RNase activity that degrades the mRNA at sites not bound by the siRNAs. The synthesis of the protein encoded by the mRNA targeted by the siRNAs is prevented, and that protein is selectively depleted from the cell. RNAi-mediated silencing can be induced experimentally by introducing synthetic siRNAs into cells using various transfection methods including liposomes (bottom left). Viral vectors can also be used to express dsRNAs against a specific gene, which are then acted upon by Dicer.

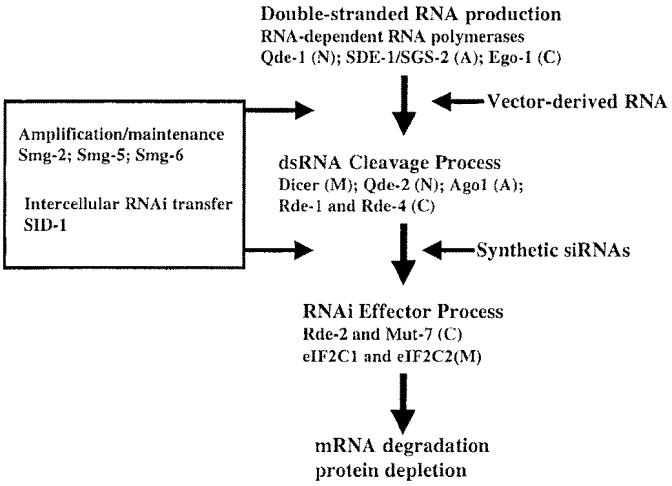

FIG. 2. Proteins involved in the process of RNAi. The production of dsRNA from a single-stranded RNA (ssRNA) template is mediated by RNA-dependent RNA polymerases such as Ode-1, SDE-1, and Ego-1. The cleavage of dsRNA to produce siRNAs is mediated by Dicer and related proteins such as Ode-2, Ago1, Rde-1, and Rde-4. The components of the RISC complex that mediate the recognition and degradation of the mRNA targeted by the siRNAs may include Rde-2, Mut-7, eIF2C1, and eIF2C2. In addition to this intrinsic RNAi pathway, mechanisms exist for amplification of RNAi (the Smg-2, Smg-5, and Smg-6 proteins appear critical for this process in *C. elegans*) and intercellular transfer of RNAi (the protein SID-1 may play a key role in this process in *C. elegans*). A, *Arabidopsis*; C, *C. elegans*; M, mammals; N, *Neurospora*.

et al., 2000) suggesting that it plays a role in the production of the RNAi signal. Another set of homologous genes involved in RNAi, identified in the screens for PTGS/RNAi mutants, include *ego-1* in *C. elegans* (Tabara et al., 1999; Smardon et al., 2000), *qde-1* in *Neurospora* (Cogoni and Macino, 1999) and *sgs2/sde1* in *Arabidopsis* (Mourrain et al., 2000). The latter proteins appear to be required for PTGS and may act by catalyzing the production of dsRNA in cells because they contain motifs similar to those of RNA-directed RNA polymerases that convert a single-stranded RNA template into a dsRNA. Further studies in *C. elegans* have identified the *smg-2*, *smg-5*, and *smg-6* genes as being involved in PTGS. However, the products of these genes are not required for the initial silencing by dsRNA, but are required for long-term maintenance of the gene suppression (Domeier et al., 2000). Smg-2 apparently amplifies the RNAi signaling such that it persists for the lifetime of the worm, whereas SMG-5 and SMG-6 are phosphatases that may facilitate Smg-2's actions by dephoshorylating it. Interestingly, Smg-2 shares a high degree of homology with yeast UPF1, a protein known to have RNA binding, ATPase and helicase activities (Page et al., 1999).

A working model for RNAi is shown in Figs. 1 and 2. The first step is the production of dsRNA directed against an mRNA. The second step involves the recognition of dsRNA and its processing to produce 21–23 nucleotide siRNAs. The "effector step" is the recognition of the target mRNA by the siRNAs and the selective degradation of that mRNA. The introduction of dsRNA into cells, whether produced endogenously from exogenous plasmids or viral vectors, results in its recognition by an enzyme that cleaves the dsRNA into 21- to 23-nucleotide double-stranded fragments in an ATP-dependent, processive manner with a 2-nucleotide 3′-overhang and a 5′-phosphorylated end (Zamore et al., 2000; Elbashir et al., 2001b). This nuclease was identified as an enzyme called Dicer that is highly conserved among plants, fungi, worms, flies, and mammals; it is a member of the RNase III family of dsRNA-specific ribonucleases (Bernstein et al., 2001). Dicer enzymes recognize and process dsRNA (Bernstein et al., 2001; Ketting et al., 2001) and are essential for RNAi (Bernstein et al., 2001; Grishok et al., 2001; Ketting et al., 2001). Dicer is thought to function as a dimer based upon knowledge of bacterial RNase III and structural evidence; crystallographic and modeling studies of RNase III suggest a mechanism for double-stranded RNA cleavage (Blaszczyk et al., 2001). Dicer not only processes dsRNA into siRNAs, but also processes endogenous regulatory RNAs called micro-RNAs. The *C. elegans* RNAi pathway gene *rde-4* encodes a dsRNA binding protein that interacts during RNAi with RNA identical to the trigger dsRNA; RDE-4 protein also interacts with Dicer and a conserved DExH-box helicase (Tabara et al., 2002). These and additional data obtained by the authors in the latter study suggest that RDE-4 and RDE-1 function together to detect, retain, and present dsRNA to Dicer for processing. Different domains of Dicer have been identified including a dsRNA binding domain, an RNase III activity domain, a helicase activity domain and a PAZ domain (Piwi-Argonaut-Zwille domain, a region of a hundred amino acids, which could mediate interaction with argonaute proteins) (Bernstein et al., 2001). Mouse Dicer is very similar to human Dicer with a predicted size of 1906 amino acids and molecular mass of 215 kDa, and contains a tandem repeat of RNase III catalytic domains, dsRNA binding region, a DExH/DEAH helicase motif and a PAZ domain (Nicholson and Nicholson, 2002). The mouse Dicer gene is located in chromosome 12 and the gene is widely expressed in cells throughout the body in embryonic and adult life.

Once generated, the small 21–23 nucleotide dsRNA fragments called siRNA are then recognized by a multiprotein complex called RISC and used as a guide for the recognition and degradation of the target mRNA (Tuschl et al., 1999; Hammond et al., 2000; Zamore et al., 2000; Nykanen et al., 2001). Experiments in *Drosophila* showed that RISC is present as a precursor complex that can be activated by ATP to form a complex with endonuclease activity that can cleave endogenous mRNAs (Hammond et al., 2000, 2001; Nykanen et al., 2001). The specific components of the RISC are not known, but do include members of the Argonaute family (Hammond et al., 2001) that have been implicated in many processes previously linked to post-transcriptional silencing. Moreover, RISC should include protein responsible for

endo-and *exo*-nuclease activity. Recently, RISC activity was studied in a human model. Two proteins of the Argonaute family, eIF2C1 and eIF2C2, were identified in the affinity-purified human RISC; the authors further showed that RISC uses single-stranded siRNAs as a guide to cleave the endogenous mRNA. In their studies of the mechanism of RNAi in human cells, Chiu et al. (2002) provided evidence that the status of the 5′-hydroxyl terminus of the antisense strand of a siRNA determines RNAi activity, whereas blocking the 3′ terminus does not prevent RNAi. They found that an A-form helix structure was required for the formation of antisense-target RNA duplexes. Surprisingly, RNAi still occurred when the siRNA duplex was cross-linked by psoralen, suggesting that complete unwinding of the siRNA helix is not necessary for RNAi activity. Thus, it appears that amplification of RNA by RNA-dependent RNA polymerase is not essential for RNAi in human cells.

It is likely that additional proteins modify the different steps in the RNAi process. For example, recent experiments have shown that the *Drosophila* homolog of the fragile X mental retardation protein interacts with Dicer and RISC suggesting a possible role in the RNAi machinery (Caudy et al., 2002; Ishizuka et al., 2002). The latter results also raise the possibility of a role of abnormalities in RNAi in various human diseases.

C. Other Related Phenomena

In addition to producing dsRNAs, which are cut into siRNAs and then (together with proteins of the RISC complex) target and degrade an mRNA species, some cells possess additional mechanisms for post-transcriptional gene regulation at the RNA level. Cells contain large amounts of noncoding RNA including tRNAs, snRNAs and rRNA (for review, see Eddy, 2001). Among these, a particular class called micro-RNA (miRNA) has recently received considerable attention. MiRNAs are approximately 22 nucleotides in length and are present in many different organisms from *C. elegans* to humans. Studies of the *lin-4* gene in *C. elegans* have demonstrated that miRNAs are able to block the translation of specific mRNAs from the *lin* family by binding to the 3′-untranslated region. In contrast to siRNA, the mRNA targeted by the miRNA is not destroyed during this process. First expressed as a 70-nucleotide stem loop precursor, lin-4 RNA is further processed by the same Dicer protein involved in siRNA-mediated RNAi. After processing the precursor, lin-4 RNA can bind on the target RNA region by complementary base pairing. The synthesis of lin-14 and lin-28 proteins is repressed by this miRNA mechanism to control development of the worm. A recent study showed that in human cells both siRNAs and miRNAs function concomitantly in the process of PTGS (Hutvagner and Zamore, 2002). The expression of some miRNAs, such as lin-4, are tightly regulated over time and seem to play an important role in development. Such temporal regulation has only been established for some of the miRNAs discovered so far; such RNA species are called small temporal RNA, which can be considered a subset of miRNA (Banerjee and Slack, 2002). It was recently shown that, as with siRNAs, miRNAs can be used as a tool to suppress expression of genes of interest (McManus et al., 2002).

Nonsense-mediated mRNA decay (NMD), although not strictly a PTGS phenomenon, is relevant to the general topic of RNAi. NMD is a process that appears to be a quality control mechanism that eliminates nonsense transcripts such as mRNAs with premature termination codons (Frischmeyer and Dietz, 1999). First discovered in yeast, this surveillance mechanism is ubiquitous among eukaryotes. Coupled to mRNA splicing, this pathway results in the degradation of aberrant mRNAs. There is evidence that NMD is involved in the PTGS-related degradation of the mRNA, because some *C. elegans* genes are required for both RNAi and NMD (Domeier et al., 2000). The two mechanisms are different, however, because NMD is dependent on translation of the mRNA, whereas the decrease in mRNA observed in RNAi and related PTGS phenomena is not prevented by inhibitors of translation (Holtorf et al., 1999). Also, the mRNA degradation associated with NMD begins with de-capping followed by 5′ to 3′ exonuclease degradation (Ruiz-Echevarria et al., 1996), whereas the degradation associated with PTGS appears to begin with endonucleotidic cleavage (Elbashir et al., 2001b).

III. Technical Considerations in the Use of RNA Interference

In several respects the approaches for silencing gene expression using RNAi methods are similar to those used for antisense DNA-mediated suppression of gene expression. In principle, any cloned gene can be targeted by designing RNA oligonucleotides or RNA-expressing viral vectors with sequences complementary to the mRNA transcribed from the target gene. The present section of this review article is intended to provide readers who are planning to use, or have just begun to use, RNAi technology into their experimental tool kit with practical information on designing and performing experiments using RNAi methods. In addition, we provide descriptions of emerging technical approaches for selective gene silencing in vitro and in vivo using RNAi. Examples of studies that employed methods described in the section can be found in Table 1. More detailed information on technical aspects of RNAi technology can be found on several different websites including: http://www.ambion.com; http://www.imgenex.com; http://www.genetherapysystems.com.

A. Design and Synthesis of Small Interfering RNAs

Double-stranded RNAs of 20–23 nucleotides (siRNAs), which can be synthesized in large quantities

TABLE 1
Applications of RNA interference

Gene	Organism	In vitro/In vivo	Cell Line/Tissue	RNA Species	Delivery Method	Reference
Acyl-CoA binding protein	Human	in vitro	HeLa, HopG2, Chang Cells	slRNA	Oligofectamine	Faergeman and Knudsen, 2002
Androgen receptor (polyCAG)	Human	in vitro	HEK293T cells	slRNA	CellFectin	Caplen et al., 2002
β-galactosidasa	Mouse	in vivo	Embryo	slRNA	Microinjection/ electroporalion	Calegari et al. 2002
β-tubulin	Mouse	in vitro	P19 cells	slRNA, shRNA (transcribed) shRNA (expressed)	Llpofectamine 2000	Yu et al. 2002
CD4, p24	Human, Virus	in vitro	Magl-CCR5, HoLa-CD4 cells	slRNA	Oligofectamine	Novins et al. 2002
CD4/CD8α	Mouse	in vitro	T lymphocytes primary T cells and bone marrow derived dentritic cells, hematopoietic stem cells, ES cells, zygotes, adult mice	slRNA	Electroporation/ cationic lipid	McManus et al. 2002
CD8, CD25, Bim, p53	Mouse	in vitro/in vivo		shRNA (U6 promoter-driven)	Lentivirus	Rubinson et al. 2003
Cdc14a	Human	in vitro	HeLa cells	siRNA	Oligofectamine	Mailand et al. 2002
Cdc14A	Human	in vitro	HeLa cells	siRNA	Oligofectamine	Mailand et al. 2002
CDH1, p53	Human	in vitro	MCF-7	shRNA (H1 promoter)	Retrovirus	Brummalkamp et al. 2002
Centrin-1	Human	in vitro	HeLa cells	siRNA	Oligofectamine	Salisbury et al. 2002
Cep135	hamster	in vitro	CHO cells	siRNA	Lipofectamine, Fugene	Ohta et al. 2002
Dicer	Human	in vitro	HEK293T cells	shRNA plasmid	Calcium phosphate	Paddison et al. 2002[b]
DNA-PK	Human	in vitro	Fibroblesis	siRNA	Liposomes	Peng et al. 2002
Drep1	Human	in vitro	HEK293T cells	shRNA plesmid	Calcium phosphate	Irotni et al. 2002
Fortilin, mycloid cell leukemia 1 protein	Human	in vitro	U2OS	siRNA	Transit solution	Zhang et al. 2002
GalTIIA, GalTIIB	Human	in vitro	HeLa	siRNA	Oligofectamine	Bal et al. 2001
GFP	Human	in vitro	293T	shRNA construct	Lentivirus	Abbas-Terki et al. 2002
GFP	Mouse	in vivo	Brain/striatal cells	shRNA	Adenovirus	Xia et al. 2002
GFP	Mouse	in vivo (mouse)	Whole organism	shRNA (H1 promoter)	Lentivirus	Tiscomia et al. 2003
GFP, luciferase, β-catenin	Human	in vitro	HeLa S3/EBNA-1	shRNA (U6 promoter-driven)	Lipofectamine 2000	Miyagishi and Taira, 2002
GFP, mAbp1, Dok2	Human	in vitro	293T	siRNA	Lipofectamine	Mise-Omata et al. 2003
GFP, MAP-2	Rat	in vitro	Primary cortical neurons	siRNA	Lipofectamine 2000	Krichevsky and Kosik, 2002
GFP, PKR	Human (PKR)	in vitro	HeLa, HEK293	siRNA (T7 promoter-generated)	Lipofectamine, calcium phosphate	Donze and Picard, 2002
Hepatitis C-NS5B	Virus	in vitro	Huh-7.5	siRNA	Electroporation	Randall et al. 2003
HIV-1 rev	Virus	in vitro	293/EcR cells	shRNA (U6 promoter-driven)	Lipofectamine Plus	Lee et al. 2002
HIV-gag	Virus	in vitro	HEK293/T lymphocytes	siRNA/fluorine derivatized siRNA	Lipofectin or naked addition (derivatized)	Capodici et al. 2002
Human papillomavirus genes E6 and E7	Virus	in vitro	Cervical carcinoma cells	siRNA	Oligofectamine	Jiang and Milner, 2002
Karyopherin α2, α3	Porcine	in vitro	Embryo	siRNA	Microinjection	Cabol and Prether, 2003
Kinase interacting stathmin	Human	in vitro	HEK 293 cells	siRNA	Lipofectamine 2000	Boehm et al. 2002
Kinesin E15	Human	in vitro	HeLa	siRNA	Calcium phosphate, oligofectamine electroporation	Weil et al. 2002
Lamin A/C	Human	in vitro	HeLa cells	shRNA (U6 promoter-driven)	Lipofectin Plus	Paul et al. 2002

PI3 kinase, phosphatidylinositol 3-kinase

TABLE 1—*Continued*

Gene	Organism	In vitro/In vivo	Cell Line/Tissue	RNA Species	Delivery Method	Reference
Luciferase		in vitro	293T/Cos1/NIH3T3/HeLe	shRNA plasmid	Calcium phosphate	Paddison et al. 2002a
Luciferase		in vivo (mouse)	Liver, kidney, spleen, lung, pancreas	siRNA	Tail vein injection	Lewis et al. 2002
Luciferase		in vivo (mouse)	Liver	siRNA	Hydrodynamic transfection, naked RNA	McCaffrey et al. 2002
Luciferase, famin A/C	Human (lamin A/C)	in vitro	HeLa, Cos-7, HEK293, HEK293	siRNA	Oligofectamine	Elbashir et al. 2002a
mAPH-1a and b, nicastrin, presenilin	Human	in vitro	HeLa cells	siRNA	Oligofectamine	Lee et al. 2002
Mina53	Human	in vitro	HeLa, rat3Y1MycB cells	siRNA	Oligofectamine	Tsuneoka et al. 2002
Mps1 kinase	Human	in vitro	HeLa cells	siRNA	Oligofectamine	Stucke et al. 2002
Myocyte enhancer factor 2A	Mouse	in vitro	Primary cerebellar granulo neurons	shRNA	Calcium phosphate	Gaudilliere et al. 2002
Nuclear Obf2-related kinase, p75	Human	in vitro	HeLa cells	shRNA	Retrovirus	Devroe and Silver, 2002
Nuclear, cytoskeletal, cell cycle	Human	In vitro	HeLa	siRNA	Oligofectamine	Harborth et al. 2001
Oct 3/4, c-mos	Mouse	in vitro	Oocytes	siRNA	Microinjection	Kim et al. 2002
Oml/Htre2	Human	in vitro	U2Os	siRNA	Effectene	Martins et al. 2002
Orc6	Human	in vitro	HeLa cells	siRNA	Liposomes	Presanth et al. 2002
p110 subunit of P13 kinase, akt1, akt2	Human	in vitro	HeLa	shRNA plasmid; siRNA	Effectene, oligofectamine	Czaudema et al. 2003
p21	Mouse	in vitro	Prostate cancer cell line	siRNA	Liposomes	Ukomadu and Dutta, 2003
p53	Human	in vitro	HEK293T cells	shRNA	Lentivirus	Barton and Medzhhitov, 2002
p53	Human	in vitro	H1299 cells	siRNA	Lipofectamine	Martinez et al. 2002
p53	Human	in vitro	SK-N-SH cells	siRNA	Oligofectamine	Kartasheva et al. 2002
p65 subunit of NF-κB	Human	in vitro	MAGI/Jurkat cells	siRNA	Oligofectamine	Surabhi and Gaynor, 2002
PKC α	Human	in vitro	HEK293T cells	siRNA and shRNA	Cationic lipid	Leirdal and Sioud, 2002
Polio-specific capsid, polymerase	Virus	in vitro	HeLa S3, mouse embryonic fibroflasts	siRNA	Lipofectamine 2000	Gitlin et al. 2002
Polio-like kinase	Human	in vitro	HeLa cells	siRNA	Oligofectamine	Liu and Erikson, 2002
Poly (ADP-ribose) polymerase	Mouse, Rat	in vitro	Neuro 2A-derived cell line	siRNA	Lipofectamine	Gan et al. 2002
PRC1	Human	in vitro	HeLa cells	siRNA	Oligofectamine	Mollinari et al. 2002
rent1/hUpf1 and rent2/hUpf2	Human	in vitro	HeLa cells	siRNA	Liposomes	Mendell et al. 2002
RNA-specific adenosine deaminase-1	Human	in vitro	Huh-7	siRNA	Lipofectamine 2002	Wong and Lazinski, 2002
Shc	Human	in vitro	HeLa cells	siRNA	Oligofectamine	Kisielow et al. 2002
Speedy (Spy)	Human	in vitro	HEK 293T cells	siRNA	Calcium phosphate	Porter et al. 2002
Sphingosine kinase-1	Human	in vitro	HUVE cells	siRNA	Oligofectamine	Ancellin et al. 2002
Various PKC isoforms	Human, Rat	in vitro	HeLa, Cos-7, HEK293, rat-1	siRNA	Oligofectamine	Irle et al. 2002

PI3 kinase, phosphatidylinositol 3-kinase

and transfected into cells, are the most commonly used reagents for RNAi in cultured cells. All that is needed to implement siRNA-mediated silencing of expression of a gene of interest is the cDNA sequence of that gene, and commercially available reagents with which to perform the synthesis. Although targeting of siRNAs to any region of an mRNA would be expected to induce degradation of the mRNA and therefore abolish production of the encoded protein, empirical data suggest that the probability of achieving selective silencing can be increased by targeting the siRNAs to specific regions of the mRNA. Ambion (Houston, TX) recommends the following approach for designing an siRNA. 1) Beginning with the AUG start codon of the target gene transcript, scan downstream for AA dinucleotide sequences; each AA and the 3′ adjacent 19 nucleotides are potential siRNA targets. 2) Compare the sequences of the potential target sequences to sequences in the species-appropriate genome database (www.ncbi.nlm.nih.gov/BLAST/) and eliminate from consideration any target sequences that are homologous to other coding sequences. 3) Select 3–4 target sequences along the length of the gene for production of siRNAs. Of course it is important for all siRNA experiments to include negative control siRNAs with the same nucleotide composition but a scrambled sequence. See http://www.mpibpc.gwdg.de/abteilungen/100/105/public.html for further information.

Chemical synthesis was the first method used to produce siRNAs, but now they can be produced in any laboratory using in vitro transcription methods. One protocol involves the synthesis of DNA oligonucleotides that include an 8-base sequence complementary to the 5′ end of a T7 promotor primer. Each gene-specific oligonucleotide is annealed to the T7 promoter primer, and a fill-in reaction using Klenow fragment produces a double-stranded template for use in an in vitro transcription reaction (Ambion Silencer siRNA construction kit). The two RNA products of the in vitro transcription reactions are hybridized to each other, treated with DNase (to remove the DNA template) and RNase (to even the ends of the dsRNA), and the RNA is column purified. Another protocol for the production of siRNAs takes advantage of the availability of recombinant human Dicer. Large in vitro transcribed RNA templates are cleaved by Dicer to produce multiple species of 22 base pair siRNAs (Dicer siRNA generation kit; Gene Therapy Systems Inc., Dan Diego, CA). An advantage of the latter method is that, because it produces a mixture of different siRNAs directed against the same mRNA target, the probability of obtaining gene silencing is increased.

B. Construction of Plasmids and Viral Vectors for RNA Interference

There are several reasons why expression plasmids and viral vectors are being used in basic and applied RNAi research. One major reason is that expression vectors allow continuous production of siRNAs in cells and, therefore, sustained depletion of the protein encoded by the targeted mRNA. A second reason is that, particularly with viral vectors, the transfection efficiency of certain types of cells, particularly postmitotic cells can be greatly increased. A third advantage of viral vectors is that they are typically more effective in obtaining sustained expression (and gene silencing, in the case of RNAi) in vivo. For example, adenoviral vectors have been extensively used to express genes in postmitotic neurons in vivo (Smith and Romero, 1999).

Short hairpin RNAs (shRNAs) can be transcribed from RNA polymerase III promoters in cells in culture or in vivo allowing continuous suppression of expression of the targeted mRNA (Paddison et al., 2002a). The latter authors proposed the use of this technology in the generation of transgenic mice as an alternative approach to gene knockout mice. Brummelkamp and colleagues (Brummelkamp et al., 2002a) developed a novel vector system for the stable expression of siRNAs in mammalian cells. They used the polymerase-III H1-RNA gene promoter, which produces a small RNA transcript lacking a poly-adenosine tail and has a well defined transcription start and termination signals. The construct also allows cleavage of the transcript at the second uridine after the termination resulting in a transcript that resembles the ends of synthetic siRNAs. They designed a gene-specific insert that specified a 19-nucleotide sequence derived from the target transcript, separated by a short spacer from the reverse complement of the same 19-nucleotide sequence resulting in the production of a 19-base pair stem-loop structure. This vector system was shown to be effective in sustained suppression of target gene expression in several different types of cultured cells.

Several laboratories have constructed plasmids that contain DNA templates for the synthesis of siRNAs under the control of the U6 promoter. For example, Sui et al. (2002) inserted DNA fragments that acted as templates for the synthesis of small RNAs under the control of the mouse U6 promoter that directs the synthesis of a Pol III-specific RNA transcript to generate an RNA composed of two identical 21-nucleotide sequence motifs in an inverted orientation, separated by a 6-base pair spacer of nonhomologous sequences. Five thymidines that function as a termination signal for Pol III were added at the 3′ end of the repeat; the resulting RNA is predicted to fold back to form a hairpin dsRNA with a 3′ overhang of several thymidines. Using this plasmid,

they were able to demonstrate the efficient inhibition of expression of three different endogenous genes (lamin A/C, CDK-2, and DNA methyltransferase) in cultured human cells. A similar approach that employed U6 promoter-driven siRNAs with four uridine 3′ overhangs was used to effectively suppress expression of ectopically expressed genes as well as the endogenous β-catenin gene (Miyagishi and Taira, 2002). Retroviral delivery systems have been developed based upon several commercially available vectors. For example, a retroviral siRNA vector was developed in which the U6 promoter and anti-target gene hairpin was subcloned into pMSCVpuro (BD Biosciences Clontech, Palo Alto, CA) at the unique NsiI site just upstream from the 3′ long terminal repeat (Devroe and Silver, 2002). Using this retroviral siRNA delivery system, they demonstrated the efficient and sustained depletion of the NDR kinase and the transcriptional coactivator p75 in cultured cells.

Lentiviral systems for shRNA delivery have also been developed. Lentiviruses can infect noncycling and postmitotic cells, and also provide the advantage of not being silenced during development allowing generation of transgenic animals through infection of embryonic stem cells or embryos (Naldini, 1998; Lois et al., 2002; Pfeifer et al., 2002). Using this approach, silencing of green fluorescent protein (GFP) in GFP-positive transgenic mice has been shown after transduction with lentiviruses expressing shRNA directed against the GFP protein (Tiscornia et al., 2003). More recently, Rubinson et al. (2003) used lentivirus-delivered shRNA to induce silencing of CD8 and CD25 in cycling primary T cells and the pro-apoptotic molecule Bim in primary bone marrow-derived dentritic cells. Lentiviral-mediated silencing of CD8 in hematopoietic stem cells was still present after injection of the cells in lethally irradiated congenic mice. Moreover, in vivo silencing for CD8 or p53 was also observed after infection of ES cells or zygotes leading to stable and functional silencing in adult RNAi transgenic mice.

C. Transfection Methods

Several different transfection methods previously used to introduce oligodeoxynucleotides and DNA plasmids into cells have been used to successfully introduce siRNAs into cells. However, it has become clear there is no single transfection method that can be successfully applied to all cell types under all experimental conditions. It is therefore important to optimize transfection conditions so that maximum gene silencing is acheived. The following transfection parameters have been shown to affect transfection and gene silencing efficacy: cell culture conditions, including cell density and medium composition; the type and amount of transfection agent; the quality and amount of siRNA; and the length of time that the cells are exposed to the siRNA. For proliferating cells, a subconfluent cell density is preferable. For postmitotic cells such as neurons, cell densities in the range of 200 to 500 cells per mm^2 of culture surface work well (O. Milavet and M. P. Mattson, unpublished data). Because proteins in serum can bind to and/or degrade siRNAs, the transfection should be performed in serum-free medium. Differences have been reported in the ability to transfect and silence gene expression between adherent and nonadherent cells. For example, the ErbB3 gene was readily silenced in adherent carcinoma cells using liposome-mediated siRNA transfection, whereas the same transfection method was ineffective in nonadherent myeloma cells (Walters and Jelinek, 2002). Postmitotic cells such as neurons and muscle cells tend to be more difficult to transfect using liposomes compared to mitotic cells such as stem cells, fibroblasts, and tumor cells.

Calcium phosphate-mediated transfection has been used successfully by several laboratories (Donza and Picard, 2002; Weil et al., 2002). The most commonly used and effective transfection method for short-term suppression of gene expression using RNAi is to incorporate siRNAs into liposomes. There are an increasing variety of such transfection reagents including: Oligofectamine, LipofectAMINE-2000 and CellFectin from Invitrogen (Carlsbad, CA) (Caplen et al., 2002; Gan et al., 2002; Gitlin et al., 2002; Irie et al., 2002; Wong and Lazinski, 2002; Mise-Omata et al., 2003); Effectene from Qiagen (Valencia, CA) (Martins et al., 2002); and siPORT-Amine and siPORT-Lipid from Ambion (Austin, TX). Other methods that have proven effective for transfecting siRNAs into cultured cells include electroporation (Calegari et al., 2002; McManus et al., 2002; Randall et al., 2003), microinjection (Calegari et al., 2002; Kim et al., 2002), and hydrodynamic shock (McCaffrey et al., 2002). Similar transfection methods have been used to introduce RNA-expressing plasmids into cultured cells (Iratni et al., 2002; Czauderna et al., 2003). An example of the kind of results obtained with an optimized transfection protocol that employed Oligofectamine is shown in Fig. 3 where levels of the cellular prion protein are markedly decreased in mouse neural precursor cells using two different siRNAs.

Xia and coworkers (Xia et al., 2002) described the development of methods for using a viral-mediated delivery system for gene silencing in mice in vivo. They constructed a 21-base pair hairpin representing sequences directed against enhanced green fluorescent protein (eGFP), and tested its ability to reduce target gene expression in mammalian culture cells. Two different constructs were used, one in which the siRNA hairpin targeted against eGFP was placed under the control of the cytomegalovirus promoter and contained a full-length simian virus-40 (SV40) polyadenylation (polyA) cassette, and the second in which the hairpin was juxtaposed almost immediate to the cytomegalovirus transcription start site (within 6 base pairs) and was followed by a synthetic, minimal polyA cassette. Cotransfection of these constructs with an eGFP expres-

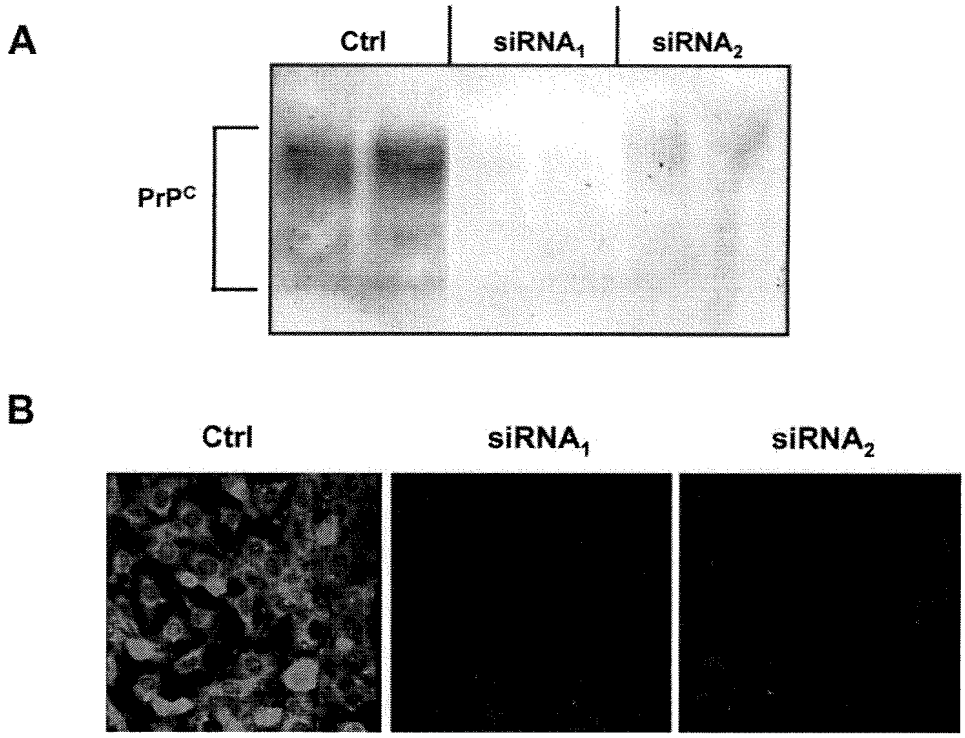

FIG. 3. Selective depletion of the cellular prion protein in neural progenitor cells by siRNA-mediated post-transcriptional gene silencing. Two chemically synthesized siRNAs were designed to target two different regions of the cellular isoform of the prion protein (PrP^C). siRNAs (50 nM) were transfected into cultured C17.2 mouse cerebellar neural progenitor cells using Oligofectamine reagent. Five days after transfection, cell lysates were subjected to immunoblot analysis using an antibody against PrP^C (panel A). Additionally, cells were fixed and immunostained with a PrP^C specific antibody before analysis by confocal microscopy (panel B). Note that both siRNAs greatly reduced the amount of PrP^C in the cells, compared to mock-transfected control cells.

sion plasmid showed that the second construct was effective in silencing eGFP expression (silencing was correlated with the generation of a 63-base pair RNA specific for eGFP). The authors then constructed recombinant adenoviruses that expressed siRNAs directed against either GFP or β-glucuronidase. These vectors were effective in suppressing expression of endogenous GFP (in GFP transgenic mice) and β-glucuronidase in liver or brain in vivo (Xia et al., 2002). Another study reported the use of a rapid injection method to deliver a large volume of physiological solution containing siRNAs into the tail vein of mice (Lewis et al., 2002). They demonstrated the effectiveness of this method for reducing target gene expression in cells throughout the body by coinjecting postnatal mice with 10 ug of a plasmid containing the luciferase gene along with 5 ug of a synthetically prepared siRNA duplex targeted against luciferase (or control siRNAs). One day after injection they collected several different organs, prepared homogenates, and screened them for luciferase activity. Luciferase activity was decreased by 80 to 90% in the liver, spleen, lung, kidney, and pancreas of mice injected with luciferase siRNA, compared with that in mice injected with control siRNAs. They further showed that inhibition of target gene expression by siRNA was dose-dependent and persisted for several days after siRNA administration. Using similar approaches, it was shown that gene expression can be suppressed in adult mice by synthetic small interfering RNAs and by small-hairpin RNAs transcribed in vivo from DNA templates (McCaffrey et al., 2002). The latter study also demonstrated the therapeutic potential of RNAi by suppressing expression of a sequence from the hepatitis C virus in the mice. The methods used to transfect cells with viral vectors that produce shRNAs are essentially identical to those used to transfect cells with similar vectors designed to express cDNAs (Abbas-Terki et al., 2002; Barton and Medzhhitov, 2002; Devroe and Silver, 2002; Xia et al., 2002). In another study, injection of liposomes containing siRNAs directed against the mRNA-encoding agouti-related peptide, a peptide known to regulate body weight, resulted in an increase in metabolic rate and reduced body weight without a change in food intake (Makimura et al., 2002).

It should be recognized that as RNAi technology advances it will likely be possible to produce RNAi "knockout" mice (or other mammals) in which the expression of a protein of interest is repressed by the expression of its corresponding RNAi related molecule (shRNA or miRNA, for example). The resulting animal could be seen as an equivalent of its knockout generated by targeted gene disruption but with much more flexibility and efficiency. For example, cell type-specific promoters could be used to effect PTGS only in cells of interest; in

many cases this may circumvent embryonic lethality resulting from gene deletion from all cells. It would also be quicker and less costly to produce RNAi transgenic animals compared with conventional knockouts.

IV. Applications of RNA Interference to Establishing Gene Function

The most widely used RNAi technology has been in cell culture and in vivo studies aimed at understanding the function of an individual (or multiple) proteins. The kinds of studies described below and listed in Table 1 demonstrate the power and flexibility of RNAi for unambiguously establishing (or excluding) a function of individual proteins in various cellular processes. It should also be noted that cells can be transfected with different combinations of multiple siRNAs, each directed against a specific mRNA of interest, to elucidate the specific contributions of the proteins to a biological process involving a multiprotein complex. For example, Wojcik and DeMartino (2002) recently took advantage of the latter feature of RNAi methods to elucidate roles for different subunits of the proteasome in its assembly and function. The power of *C. elegans* and *Drosophila* molecular genetics is providing the opportunity to use genome-wide RNAi to rapidly establish functions of genes in a specific process. For example, Ruvkun and colleagues (Lee et al., 2002c) systematically inactivated 5690 genes in *C. elegans* using RNAi to identify genes that limit lifespan. They identified a mitochondrial leucyl-tRNA synthetase gene and showed that mutations of this gene that impair mitochondrial function increase lifespan, which was associated with decreased ATP levels and oxygen consumption.

A. Signal Transduction

Intercellular messenger molecules play vital roles in the development and proper functioning of all organisms. Among the most prominent of such signals in mammals are growth factors, cytokines, cell adhesion molecules, neurotransmitters, steroids, and gases such as nitric oxide. Specific receptors located on the cell surface or within the cell transduce responses to the ligand via signaling cascades that are often complex, involving kinases and transcription factors, for example. RNAi methods provide powerful tools for establishing the roles of individual proteins in the signal transduction pathway employed by a specific ligand. Several recent studies have demonstrated the efficacy of siRNA-mediated knockdown of signal transduction proteins and have elucidated roles for those proteins in biological responses of cells. Adaptor proteins of the Shc family transduce signals from a diverse group of growth factors that signal through receptor tyrosine kinases. Liposome-mediated introduction of siRNA against a single isoform of ShcA into HeLa cells was used to selectively reduce levels of that Shc revealing its role in the regulation of cell proliferation (Kisielow et al., 2002). Neurotrophin receptors and integrins (receptors for extracellular matrix molecules such as laminin) are often coupled to a signaling pathway involving phosphatidylinositol 3-kinase and Akt kinase (Gary and Mattson, 2001; Cheng et al., 2003). Decreasing the amount of the 110 β subunit of phosphatidylinositol 3-kinase using siRNAs resulted in a marked decrease in the growth and tissue invasiveness of tumor cells (Czauderna et al., 2003). Small molecule inhibitors have been widely employed to study the functions of various protein kinases in cells. However, most such inhibitors are not specific and affect multiple kinases. RNAi has been successfully employed to unequivocally establish the roles of specific kinases in signal transduction processes. For example, Irie and coworkers (Irie et al., 2002) demonstrated the ability to knockdown levels of specific subtypes of protein kinase C in cultured human and rat cells in a species-specific manner. The existence of an extracellular system for the production of sphingosine-1-phosphate was demonstrated in a study in which siRNAs directed against the sphingosine kinase-1 enzyme were used to inhibit its production and export in cultured endothelial cells (Ancellin et al., 2002).

Calcium plays important roles as an intracellular signal that mediates a variety of responses of cells to environmental stimuli. Mechanisms for regulating levels of calcium in the cytoplasm are complex and involve movements of calcium ions across the plasma membrane, as well as into and out of endoplasmic reticulum and mitochondria. A key role for inositol 1,4,5-trisphosphate (IP_3)-mediated release of intracellular calcium in the maturation of mouse oocytes was demonstrated in which siRNAs against the IP_3 receptor-1 were injected into germinal vesicle-intact oocytes (Xu et al., 2003). The siRNAs reduced IP_3 receptor-1 levels by 90% and, following insemination, blocked the intracellular calcium oscillations that play a critical role for the first steps in development. In another study, RNAi-mediated depletion of the endoplasmic reticulum calcium-ATPases resulted in lethality in *C. elegans* (Cho et al., 2000), demonstrating a pivotal role for calcium uptake by this organelle in cell functions and survival.

B. Cell Cycle Regulation

Because of their fundamental importance for development, tissue homeostasis, and stem cell biology and their centrality to the field of cancer research, genes that regulate the cell cycle have been a prominent focus of studies that employ RNAi. Examples of the kinds of findings obtained using RNAi technologies to knockdown the expression of cell cycle genes are as follows. A requirement of the Mps1 kinase for the spindle assembly checkpoint was established, whereas a role for this kinase in centrosome duplication was ruled out (Stucke et al., 2002). A key role for a novel centrosomal protein called Cep135 in microtubule organization was revealed

(Ohta et al., 2002), and a role for centrin-2 in centriole duplication was established (Salisbury et al., 2002). RNAi was used to show that the regulation of cell cycle progression in response to mitogens is controlled by the cyclin-dependent kinase inhibitor p27 (Kip1) (Boehm et al., 2002). Depletion of Plk1 using siRNAs results in activation of cyclin B and inhibits centrosome amplification in hydroxyurea-treated U2OS cells (Liu and Erikson, 2002). RNAi was used to show that the protein PRC1 is a microtubule-associated protein that facilitates bundling of microtubules (Mollinari et al., 2002) and that the processes of centrosome separation and chromosome segregation require the phosphatase Cdc14A in culture human cells (Mailand et al., 2002). Depletion of the origin recognition complex subunit ORC6 using RNAi resulted in cells with decreased DNA replication, multipolar spindles and aberrant mitosis (Prasanth et al., 2002). A novel human protein called Speedy, expressed only during the G_1/S phase of the cell cycle, was shown to enhance cell proliferation by enhancing the activity of Cdk2 (Porter et al., 2002).

C. Development

The complex and remarkably rapid events that occur during development of the fertilized egg into an adult organism remain largely a mystery. There would appear to be a great potential for RNAi technology to unravel the cellular and molecular events that regulate developmental processes. Methods for silencing single or multiple selected genes in developing embryos in vivo and stem cells in culture (see *Section III.C.*) are beginning to reveal the functions of specific proteins in developmental processes. Nodal is a secreted factor that plays a key role in the formation and patterning of the mesoderm during gastrulation. RNAi was used to demonstrate that the transcriptional corepressor DRAP1 inhibits the transcription factor FoxH1 and thereby regulates signaling by Nodal during mouse embryogenesis (Iratni et al., 2002). Depletion of karyopherins $\alpha 2$ and $\alpha 3$ in cleavage stage porcine embryos revealed different requirements of these two proteins in embryogenesis (Cabot and Prather, 2003). In another study of embryogenesis, siRNAs directed against the mRNAs encoding Oct-3/4 and c-mos resulted in depletion of the encoded proteins, and phenotypes similar to those observed in Oct-3/4 and c-mos knockout mice (Kim et al., 2002). A key role for microtubule-associated protein-2 in the regulation of dendrite outgrowth in developing brain neurons was demonstrated using siRNAs (Krichevsky and Kosik, 2002). The transcription factor myc is known to play a fundamental role in the regulation of cell proliferation. A key role for the novel myc target gene mina53 in the regulation of cell proliferation by myc was demonstrated using RNAi technology (Tsuneoka et al., 2002).

D. Macromolecular Synthesis and Degradation

Regulated synthesis of nucleic acids, proteins, and lipids, and the turnover of those macromolecules is essential for cell survival and functions. Although biochemical technologies have allowed the identification of various biosynthetic pathways and mechanisms for the degradation of proteins and other macromolecules, the functions of specific proteins in such processes are not well understood. Recent studies have employed RNAi to advance the understanding of the regulation of macromolecular synthesis and degradation. For example, depletion of galactosyltransferase II using siRNAs revealed a critical role for this enzyme in the biosynthesis of the linkage region of glycosaminoglycans (Bai et al., 2001). The regulation of the processing of RNAs transcribed from coding and noncoding regions of the genome is an active area of investigation because of the recent realization of its importance in the regulation of gene expression. Mendell and coworkers (Mendell et al., 2002) used RNAi to show that rent1/Upf1 plays distinct roles in the regulation of splicing and decay of nonsense transcripts. Transfection of cultured HeLa and HepG2 cells with siRNAs directed against the mRNA encoding the acyl-CoA binding protein resulted in cessation of cell proliferation, cell detachment, and death, demonstrating that acyl-CoA binding protein is essential for cell survival (Faergeman and Knudsen, 2002).

E. Cell Motility

The migration of cells within and among tissues, and extensions of cells such as the axons and dendrites of neurons, is central to the structural and functional organization of all tissues. Although there are a few pharmacological agents that have proven useful in studying cell motility (the actin-depolymerizing agent cytochalasin D and the microtubule-stabilizing agent taxol, for example), a detailed understanding of the molecular regulation of cell motility is lacking. Recent studies have taken advantage of RNAi methods to elucidate roles for specific proteins in regulating cell motility. Depletion of the cytoskeletal linker protein trypanin using siRNAs resulted in a loss of directional cell motility in African trypanosomes that is caused by impaired coordination of the flagellar beating (Hutchings et al., 2002). The growth and guidance of axons in developing neurons is regulated by substrate-associated and soluble ligands encountered by the axonal growth cone. RNAi-mediated depletion of the integrin-interacting protein MIG-15 resulted in a dysregulation of commissural axon navigation in *C. elegans* (Poinat et al., 2002). RNAi has also shown that although actin binding protein 1 is essential for the processes of endocytosis, it is not necessary for lamellipodia formation in human embryonic kidney cells (Mise-Omata et al., 2003). Membrane microdomains called lipid rafts and clathrin-associated domains are increasingly recognized as sites of signal transduction

and endocytosis in eukaryotic cells. Proteins critical for the function of the signaling and endocytic functions of these membrane domains are being identified using RNAi approaches. For example, RNAi methods were used to establish an essential role for a J-domain protein called auxilin in clathrin-mediated endocytosis in *C. elegans* (Greener et al., 2001).

F. Cell Death

In many tissues throughout the body, cells have a finite life span and then undergo apoptosis, a form of programmed cell death in which the cell dies in a well controlled manner and is removed from the tissue without adversely affecting adjacent healthy cells. Apoptosis also plays a key role in sculpting the cellular structure of tissues during embryonic and postnatal development (Baehrecke, 2002). Of course, abnormal cell death is a major problem in a variety of diseases, including neurodegenerative disorders such as Alzheimer's and Parkinson's diseases, and ischemic vascular conditions (Mattson, 2000). Considerable progress is being made in understanding the molecular mechanisms that regulate cell death, with the goal of identifying key targets for therapeutic intervention. Drug development efforts have resulted in several exciting classes of agents that either prevent the death of cells such as neurons, or induce selective death of cancer cells. For example, inhibitors of the pro-apoptotic protein p53 (Duan et al., 2002; Zhu et al., 2002) and caspases (Eldadah and Faden, 2000) are being developed for use in neurodegenerative disorders. RNAi might be used in lieu or in combination with such drugs.

RNAi has recently been employed to establish roles for specific genes in apoptotic and anti-apoptotic pathways. For example, a critical role for p73δ in p53-mediated apoptosis was demonstrated by showing that depletion of p73δ using siRNA prevents cell death (Kartasheva et al., 2002). Depletion of the catalytic subunit of DNA-dependent protein kinase using siRNAs increased the sensitivity of human fibroblasts to radiation-induced death because of an impaired ability to sense and repair the DNA damaged by the radiation (Peng et al., 2002). RNAi was used to establish a role for the calcium-binding protein calreticulin in necrotic cell death in *C. elegans* (Xu et al., 2001). Many cells express one or more inhibitor-of-apoptosis proteins (IAPs) that can prevent apoptosis by directly binding to and inhibiting caspases. RNAi was used to identify the serine protease Omi/HtrA2 as a mammalian XIAP-binding protein that sensitizes cells to apoptosis (Martins et al., 2002). In a study of cell death in neurons, RNAi was used to show that myocyte enhancer factor 2A is required for activity-dependent cell survival (Gaudilliere et al., 2002). In another study, RNAi was used to deplete cells of apoptosis-inducing factor (AIF), thereby preventing apoptosis (Wang et al., 2002).

Associations between expression of a gene and a particular biological process are often taken as evidence for a major role for the protein encoded by that gene in that biological process. However, correlations do not establish cause-effect relationships. A well known example of this fact is found in the record of studies of the transcription factor NF-κB. Because NF-κB was shown to be activated in several different types of cells during the process of apoptosis, it was assumed that NF-κB functioned in the cell death process. However, subsequent studies in which NF-κB activity was selectively blocked revealed that this transcription factor actually induced the expression of anti-apoptotic proteins and that cells died more readily when NF-κB function was blocked (Mattson and Camandola, 2001). Thus, in addition to revealing the function of a specific protein, RNAi can also be used to establish that a protein is not involved in a particular process

G. Viral Invasion/Replication

It is believed that endogenous RNAi mechanisms evolved, at least in part, to protect cells against infectious pathogens such as viruses. For example, by producing siRNAs directed against genes required for viral replication, the infected cells prevented propagation of the virus (Lindenbach and Rice, 2002). The development of RNAi technology has verified the importance of RNAi mechanisms in viral invasion and is revealing the underlying molecular interactions. Treatment of various types of cultured cells with siRNAs directed against both viral and cellular targets has revealed specific roles for the targeted proteins in the processes of viral invasion or replication. When cells were treated with siRNAs directed against the HIV-1 tat or reverse transcriptase genes, or against the NF-κB p65 subunit of the host cells, the expression of these viral and cellular proteins were decreased and HIV-1 replication was inhibited, demonstrating the ability of RNA interference to elucidate the biological roles of cellular and viral regulatory factors involved in the control of HIV-1 gene expression (Surabhi and Gaynor, 2002). When human and mouse cells were pretreated with siRNAs to the poliovirus genome, the replication of the virus in those cells was dramatically reduced (Gitlin et al., 2002). In another study it was shown that siRNAs can efficiently silence both cellular lamin AC and hepatitis C virus RNAs in Huh-7 hepatoma cell lines resulting in an 80-fold decrease in HCV RNA within 4 days (Randall et al., 2003). The latter study further showed that the same siRNAs could be used to almost completely eliminate the virus from cells with an established infection. The expression of the human papilloma virus E6 and E7 genes was silenced resulting in the accumulation of cellular p53 protein, transactivation of the cell cycle control p21 gene and reduced cell growth (Jiang and Milner, 2002). A final example of the use of RNAi in the discovery of mechanisms of viral infection is a study of hepatitis

delta virus (HDV) which uses a host-encoded RNA-editing machinery to express two essential proteins from the same coding sequence (Wong and Lazinski, 2002). The authors employed siRNAs to deplete either a small or long form, or both forms, of an adenosine deaminase that acts on RNA (ADAR)1. They found that editing during viral replication was only inhibited when both forms of the enzyme were depleted, demonstrating a cooperative interaction between the short and long forms of ADAR1 in viral replication.

V. Therapeutic Applications of RNA Interference

The most obvious clinical uses of RNAi are for diseases in which selective depletion of one or a few specific proteins would be expected to slow or halt the disease process in the affected cells. Ideally this would be accomplished with no or tolerable side effects. Although there are candidate gene targets for many different diseases, we will focus on four different types of diseases that are very common and for which RNAi approaches are currently being tested in preclinical studies.

A. Cancer

There are two general abnormalities in cancer cells—they exhibit dysregulation of the cell cycle resulting in uncontrolled growth and they are resistant to death as a result of abnormalities in one or more proteins that mediate apoptosis (Nam and Parang, 2003). The goals for RNAi approaches for cancer therapy are therefore to knock out the expression of a cell cycle gene and/or an anti-apoptotic gene in the cancer cells thereby stopping tumor growth and killing the cancer cells. To selectively eliminate cancer cells without damaging normal cells, the RNAi would be targeted to a gene specifically involved in the growth or survival of the cancer cell, or the siRNAs would be selectively delivered into the cancer cells.

For many years antisense oligodeoxynucleotide technology was pursued in preclinical studies of cancer therapies but with discouraging results overall (Jansen and Zangemeister-Wittke, 2002). Recent studies have clearly demonstrated advantages of RNAi methods for the growth suppression and killing of cancer cells. In one study, siRNA was shown to be greater than an order of magnitude more potent than antisense DNA in suppressing gene expression in human hepatoma and pancreatic cancer cell lines (Aoki et al., 2003). In another study four different myeloid leukemia cell lines (HL-60, U937, THP-1, and K562) were transfected with dsRNA duplexes corresponding to the endogenous c-raf and bcl-2 genes (Cioca et al., 2003). Levels of Raf-1 and Bcl-2 proteins were markedly decreased in each of the transfected cell lines; combined RNAi for c-raf and bcl-2 induced apoptosis in HL-60, U937, and THP-1 cells and increased their sensitivity to the DNA-damaging agent etoposide. Activation of tumor necrosis factor (TNF) receptors and related death receptors can induce death of some cancer cells, but may simultaneously activate pathways that promote cell survival; one protein that inhibits the TNF cell death pathway is called FLIP (FLICE-like inhibitory protein). When FLIP expression was suppressed in cancer cells using siRNAs, the cells were more sensitive to being killed when death receptors were activated (Siegmund et al., 2002).

Viral vectors have also been used to express siRNAs and inhibit cancer cell growth and tumorogenicity. For example, a retroviral vector was used to specifically and stably inhibit expression of the oncogenic K-RAS(V12) allele in human tumor cells (Brummelkamp et al., 2002b). Depletion of K-RAS(V12) resulted in loss of anchorage-independent growth and tumorigenicity. In addition to blocking the expression of normal genes that are required for cancer cell growth and survival, RNAi can be used to target specific cancer-causing mutations. For example, dsRNA was employed to target the M-BCR/ABL fusion site to kill leukemic cells with such a rearrangement (Wilda et al., 2002). Leukemic cells without BCR/ABL rearrangement were not killed by M-BCR/ABL-dsRNA. Several other studies have demonstrated efficacy of liposome-mediated or viral vector-mediated transfection of cancer cells in suppressing their growth and/or inducing their death (Zhang et al., 2002a). The next step in the development of RNAi technology for cancer therapy will be to establish methods for targeting tumor cells in vivo. Another approach might be to target genes that promote angiogenesis. Tumor cells require a rich supply of blood and achieve this by stimulating the process of angiogenesis; it may therefore be possible to inhibit tumor growth by targeting the vascular endothelial cells involved in angiogenesis. As evidence, it was shown that depletion of the crk adaptor protein using RNAi inhibited the migration of cultured vascular endothelial cells (Nagashima et al., 2002).

B. Infectious Diseases

Diseases caused by viruses and bacteria continue to be major causes of death worldwide and are an increasing concern because of the emergence of resistant strains and the potential use of infectious pathogens by terrorists (Tan et al., 2000; Franz and Zajtchuk, 2002). Currently, HIV infection has reached epidemic proportions in many African countries and also continues to be a major cause of morbidity and death among homosexuals and intravenous drug users. Other prominent infectious diseases include influenza, hepatitis, Lyme disease, and West Nile virus. Many deaths also result from bacterial infections, with pneumonia and sepsis being prominent examples. The ability of RNAi to inhibit the replication or cellular uptake of viruses and other infectious agents has been clearly demonstrated in cell culture studies and, therefore, holds promise for the treatment of human patients. The ability of HIV-1 to infect cells and replicate can be severely compromised by targeting of

viral genes using siRNAs. Examples include the suppression of HIV-1 replication in human cells transfected with siRNA directed against tat and the rev gene (Capodici et al., 2002; Jacque et al., 2002; Lee et al., 2002a; Novina et al., 2002). Transfection of human cells with siRNAs directed against different genes in the poliovirus genome resulted in resistance of the cells to infection with poliovirus (Gitlin et al., 2002). The ability of siRNAs targeting the gene encoding the death receptor Fas to protect mice from liver failure and fibrosis in two models of autoimmune hepatitis was tested by Song and colleagues (Song et al., 2003). Intravenous injection of Fas siRNA specifically reduced Fas protein levels in the livers of mice during a 10-day period. Fas siRNA treatment abrogated hepatocyte necrosis and inflammatory infiltration and markedly reduced serum concentrations of transaminases demonstrating a clear hepatoprotective effect of the siRNA therapy.

C. Cardiovascular and Cerebrovascular Diseases

Cardiovascular disease is the leading cause of death in the United States and many other industrialized countries. It most commonly results from the progressive occlusion of arteries in a process called atherosclerosis, which can ultimately culminate in a myocardial infarction or stroke. Atherosclerosis involves damage to vascular endothelial cells, local production of inflammatory cytokines, and the recruitment of macrophages to the site forming foam cells; in addition, apoptosis of foam cells and vascular smooth muscle cells occurs (Geng and Libby, 2002). The severe ischemia that occurs in heart or brain cells during a myocardial infarction or stroke results in the death of cardiac muscle cells or neurons. Although some of the cells die rapidly by necrosis, many other cells die more slowly by apoptosis; data from animal studies suggest that such cardiac myocytes and brain neurons that die by apoptosis can be saved (Mattson et al., 2000; Zhao and Vinten-Johansen, 2002).

It may be possible to use RNAi technology to intervene in the process of atherosclerosis or to reduce the damage to heart tissue and brain cells that patients suffer following a myocardial infarction or stroke. A key step in the process of atherosclerosis is the up-regulation of cell adhesion molecules in vascular endothelial cells, which play an essential role in the recruitment of macrophages to the site of endothelial damage. The production of cell adhesion molecules can be selectively suppressed in cultured cells (Jarad et al., 2002). In another study relevant to the pathogenesis of atherosclerosis, it was shown that mevastatin, an inhibitor of cholesterol synthesis, suppresses cell proliferation by inhibiting cyclin-dependent kinase-2 (Ukomadu and Dutta, 2003).

D. Neurodegenerative Disorders

Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis are examples of relatively common age-related neurodegenerative disorders that are increasing as average life expectancy increases. Each disorder is characterized by the dysfunction and death of specific populations of neurons: hippocampal and cortical neurons involved in learning and memory processes in Alzheimer's disease, dopamine-producing neurons in the substantia nigra that control body movements in Parkinson's disease, and spinal cord motor neurons in amyotrophic lateral sclerosis. Specific genetic mutations are responsible for a small percentage of cases of Alzheimer's and Parkinson's disease and amyotrophic lateral sclerosis (Hardy, 2001), whereas all cases of Huntington's disease result from mutations (polyglutamine expansions) in the huntingtin protein (Rubinsztein, 2002). Studies of patients, and of animal and cell culture models of each disease, have revealed shared biochemical cascades that result in neuronal death. Those cascades include increased oxidative stress, dysregulation of cellular calcium homeostasis and apoptosis (Mattson, 2000). There have therefore been two different strategies for preventative and therapeutic interventions in neurodegenerative disorders. One strategy is to block the disease-specific events that are believed to initiate the neurodegenerative process, whereas the second strategy targets downstream events in the neurodegenerative cascade. For example, an abnormality in the proteolytic processing of the amyloid precursor protein is believed to be a key early event in Alzheimer's disease pathogenesis, and two enzymes called β- and γ-secretases that are responsible for cleaving of amyloid precursor protein to generate the neurotoxic amyloid β-peptide are being targeted for drug development. RNAi has recently been used to identify additional proteins, such as APH-1, that are critical for production of amyloid β-peptide (Lee et al., 2002b). Downstream targets include proteins involved in the production of reactive oxygen species, in the regulation of calcium homeostasis, and in the process of apoptosis (Mattson, 2000, 2003).

Recent studies have shown that cultured neurons can be efficiently transfected with siRNAs and that the targeted genes are effectively silenced. In one study it was shown that cultured neurons can be depleted of the p75 neurotrophin receptor, a protein in the TNF receptor family that has been implicated in neuronal apoptosis in certain settings (Higuchi et al., 2003). Pro-apoptotic members of the Bcl-2 family (Colussi et al., 2000) and caspases (Quinn et al., 2000) have been effectively targeted and neuronal death prevented, using RNAi methods. Caplen and colleagues (Caplen et al., 2002) performed studies aimed at determining whether RNAi could be used to target the pathogenic process in inherited neurodegenerative disorders caused by polyglutamine expansions. They used *Drosophila* and human tissue culture models of spinobulbar muscular atrophy, a disease caused by CAG expansions in the gene encoding the androgen receptor. They assessed the abilities of different siRNAs to selectively inhibit expression of

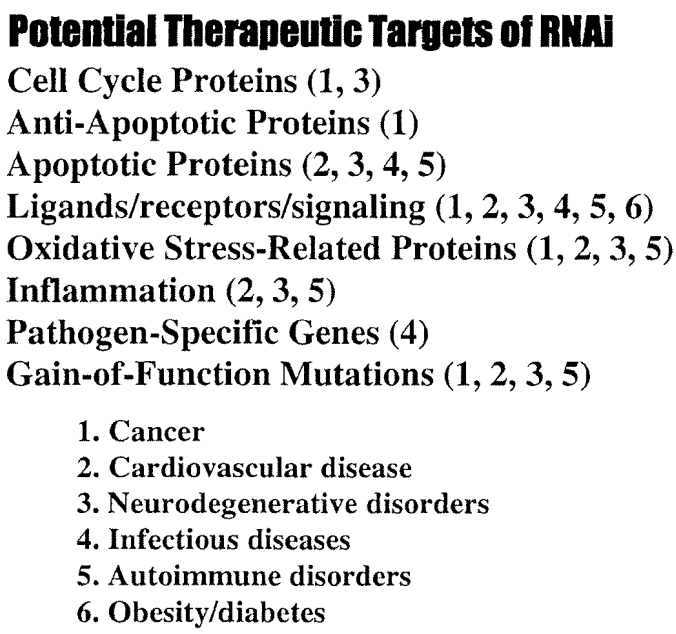

FIG. 4. Examples of therapeutic targets of RNAi in medicine. The ability to target a specific gene or genes using siRNAs or vector-mediated RNA expression methods, suggests the potential of RNAi to block the disease process or relieve symptoms of the disease. Depletion of proteins critical for the cell cycle, such as cyclins, cyclin-dependent kinases, or telomerase, might be effective in treating cancers and some neurodegenerative disorders. Blocking the production of anti-apoptotic proteins such as Bcl-2, inhibitor of apoptosis proteins and antioxidant enzymes may be used to kill cancer cells. Conversely, RNAi-mediated suppression of expression of apoptotic proteins (Bax, Par-4, p53, AIF, and caspases, for example) may slow or stop the degenerative processes in degenerative myocardial, neurological, and autoimmune disorders. Ligands, receptors, and downstream signal transduction proteins critical for a specific disease process might also be targeted. For example, it might be possible to suppress the appetite of obese patients by targeting neuropeptide Y- or ghrelin-producing cells using shRNA-expressing viral vectors. Genes that encode proteins involved in oxidative stress and inflammation (nitric-oxide synthase, cyclooxygenases, and tumor necrosis factor, for example) might be targeted in autoimmune and infectious or inflammatory diseases. Viral and bacterial genes are obvious targets for RNAi-based therapeutic intervention in infectious diseases.

transcripts that included a truncated human androgen receptor gene containing different CAG repeat lengths (16–112 repeats). They found that RNA duplexes containing CAG repeat tracts only induced gene-specific inhibition when flanking androgen receptor sequences were included. Sequence-specific small dsRNAs of 22 nucleotides rescued the toxicity and caspase-3 activation induced by plasmids expressing a transcript encoding an expanded polyglutamine tract. Thus, it is possible, at least in cell culture, to selectively silence a transcript associated with an important group of genetic diseases by RNAi.

Several aspects of neuronal cell biology provide opportunities for novel uses of RNA. Neurons possess complex morphologies with long axons and dendrites, and synapses that are often located at considerable distances from the cell body (for example, the presynaptic terminals of the axons of the lower motor neurons that innervate muscles in the foot in humans can be more than a meter from their cell bodies in the spinal cord). Recent findings suggest that the neurodegenerative cascades that occur in different neurodegenerative disorders may be activated first in synapses. Indeed, it has been shown that apoptotic biochemical cascades can be activated in synapses causing their degeneration (Mattson et al., 1998). Accordingly, pharmacological agents (p53 and caspase inhibitors; Glazner et al., 2000; Gilman et al., 2003) and antisense treatments (Par-4 antisense; Duan et al., 1999) that target apoptotic cascades have been shown to protect synapses in cell culture models of neurodegenerative disorders. RNAi technology would seem to be an ideal approach to target synaptic proteins involved in the pathogenesis of neurodegenerative disorders.

VI. The Future of RNA Interference in Biology and Medicine

Even at this early stage of understanding the molecular mechanisms of RNAi and in the development of methods for the use of RNAi technology for selective gene silencing, it is clear that RNAi will be a widely used tool for establishing the functions of genes. The ability to selectively deplete a single protein of interest in cultured cells using siRNAs, and plasmids and viral vectors, is now established. Improvements on the currently available protocols for RNAi are being made, and the methods are being applied by thousands of investigators in diverse fields. With the advent of these methods has come an explosion of studies that have employed RNAi. Indeed, although there were only nine publications listed on Medline in the years 1987 through 1998 inclusive, there are 631 listed from 1999 to the time of writing of this article. The current status of RNAi as an experimental tool is such that many investigators are now aware of the technology, but most have not yet implemented it in their own studies. The development of RNAi kits by several companies will facilitate the implementation of RNAi methods by essentially any investigator, regardless of their knowledge of RNAi mechanisms. However, major hurdles remain to be crossed, including the application of RNAi methods to in vivo studies. Once it becomes possible to reliably target a specific gene and deplete the protein encoded by that gene from one or all cell types in an organism, a wealth of information will flow from studies of processes ranging from embryogenesis to the function of organ systems.

As for uses of RNAi in medicine, its potential remains to be established. The application of gene therapy approaches for the treatment of specific diseases has progressed much more slowly than initially anticipated. There are, of course, many potential gene targets for therapeutic intervention using RNAi (Fig. 4). Studies that employ RNAi to counteract a disease process in vivo are emerging. For example, RNAi that targeted the Fas gene (Song et al., 2003) or the hepatitis C virus genome

(Wilson et al., 2003) protected mice from hepatitis. One might expect that the next demonstrations of successful treatment of disease in mice will come from models of cancer and neurodegenerative disorders. Because of the potential of RNAi for therapeutic intervention, major efforts should be placed on preclinical studies using this technology.

References

Abbas-Terki T, Blanco-Bose W, Deglon N, Pralong W, and Aebischer P (2002) Lentiviral-mediated RNA interference. *Hum Gene Ther* **13:**2197–2201.

Ancellin N, Colmont C, Su J, Li Q, Mittereder N, Chae SS, Stefansson S, Liau G, and Hla T (2002) Extracellular export of sphingosine kinase-1 enzyme. Sphingosine 1-phosphate generation and the induction of angiogenic vascular maturation. *J Biol Chem* **277:**6667–6675.

Aoki Y, Cioca D, Oidaira H, Kamiya J, and Kiyosawa K (2003) RNA interference may be more potent than antisense RNA in human cancer cell lines. *Clin Exp Pharmacol Physiol* **30:**96–102.

Assaad FF, Tucker KL, and Signer ER (1993) Epigenetic repeat-induced gene silencing (RIGS) in Arabidopsis. *Plant Mol Biol* **22:**1067–1085.

Baehrecke EH (2002) How death shapes life during development. *Nat Rev Mol Cell Biol* **3:**779–787.

Baglioni C and Nilsen TW (1983) Mechanisms of antiviral action of interferon. *Interferon* **5:**23–42.

Bai X, Zhou D, Brown JR, Crawford BE, Hennet T, and Esko JD (2001) Biosynthesis of the linkage region of glycosaminoglycans: cloning and activity of galactosyltransferase II, the sixth member of the beta 1, 3-galactosyltransferase family (beta 3GalT6). *J Biol Chem* **276:**48189–48195.

Banerjee D and Slack F (2002) Control of developmental timing by small temporal RNAs: a paradigm for RNA-mediated regulation of gene expression. *Bioessays* **24:**119–129.

Barton GM and Medzhitov R (2002) Delivery of small interfering RNA into primary cells. *Proc Natl Acad Sci USA* **99:**14943–14945.

Bernstein E, Caudy AA, Hammond SM, and Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature (Lond)* **409:**363–366.

Billy E, Brondani V, Zhang H, Muller U, and Filipowicz W (2001) Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proc Natl Acad Sci USA* **98:**14428–14433.

Blaszczyk J, Tropea JE, Bubunenko M, Routzahn KM, Waugh DS, Court DL, and Ji X (2001) Crystallographic and modeling studies of RNase III suggest a mechanism for double-stranded RNA cleavage. *Structure (Camb)* **9:**1225–1236.

Boehm M, Yoshimoto T, Crook MF, Nallamshetty S, True A, Nabel GJ, and Nabel EG (2002) A growth factor-dependent nuclear kinase phosphorylates p27(Kip1) and regulates cell cycle progression. *EMBO (Eur Mol Biol Organ) J* **21:**3390–3401.

Bosher JM and Labouesse M (2000) RNA interference: genetic wand and genetic watchdog. *Nat Cell Biol* **2:**E31–E36.

Brummelkamp TR, Bernards R, and Agami R (2002a) A system for stable expression of short interfering RNAs in mammalian cells. *Science (Wash DC)* **296:**550–553.

Brummelkamp TR, Bernards R, and Agami R (2002b) Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell* **2:**243–247.

Cabot RA and Prather RS (2003) Cleavage stage porcine embryos may have differing developmental requirements for karyopherins alpha2 and alpha3. *Mol Reprod Dev* **64:**292–301.

Calegari F, Haubensak W, Yang D, Huttner WB, and Buchholz F (2002) Tissue-specific RNA interference in postimplantation mouse embryos with endoribonuclease-prepared short interfering RNA. *Proc Natl Acad Sci USA* **99:**14236–14240.

Caplen NJ, Fleenor J, Fire A, and Morgan RA (2000) dsRNA-mediated gene silencing in cultured Drosophila cells: a tissue culture model for the analysis of RNA interference. *Gene (Amst)* **252:**95–105.

Caplen NJ, Taylor JP, Statham VS, Tanaka F, Fire A, and Morgan RA (2002) Rescue of polyglutamine-mediated cytotoxicity by double-stranded RNA-mediated RNA interference. *Hum Mol Genet* **11:**175–184.

Capodici J, Kariko K, and Weissman D (2002) Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference. *J Immunol* **169:**5196–5201.

Caudy AA, Myers M, Hannon GJ, and Hammond SM (2002) Fragile X-related protein and VIG associate with the RNA interference machinery. *Genes Dev* **16:**2491–2496.

Cheng A, Wang S, Yang D, Xiao R, and Mattson MP (2003) Calmodulin mediates brain-derived neurotrophic factor cell survival signaling upstream of Akt kinase in embryonic neocortical neurons. *J Biol Chem* **278:**7591–7599.

Chiu YL and Rana TM (2002) RNAi in human cells: basic structural and functional features of small interfering RNA. *Mol Cell* **10:**549–561.

Cho JH, Bandyopadhyay J, Lee J, Park CS, and Ahnn J (2000) Two isoforms of sarco/endoplasmic reticulum calcium ATPase (SERCA) are essential in Caenorhabditis elegans. *Gene (Amst)* **261:**211–219.

Cioca DP, Aoki Y, and Kiyosawa K (2003) RNA interference is a functional pathway with therapeutic potential in human myeloid leukemia cell lines. *Cancer Gene Ther* **10:**125–133.

Clarke PA and Mathews MB (1995) Interactions between the double-stranded RNA binding motif and RNA: definition of the binding site for the interferon-induced protein kinase DAI (PKR) on adenovirus VA RNA. *RNA* **1:**7–20.

Cogoni C, Irelan JT, Schumacher M, Schmidhauser T, Selker EU, and Macino G (1996) Transgene silencing of the al-1 gene in vegetative cells of *Neurospora* is mediated by a cytoplasmic effector and does not depend on DNA-DNA interactions or DNA methylation. *EMBO (Eur Mol Biol Organ) J* **15:**3153–3163.

Cogoni C and Macino G (1999) Gene silencing in Neurospora crassa requires a protein homologous to RNA-dependent RNA polymerase. *Nature (Lond)* **399:**166–169.

Colussi PA, Quinn LM, Huang DC, Coombe M, Read SH, Richardson H, and Kumar S (2000) Debcl, a proapoptotic Bcl-2 homologue, is a component of the Drosophila melanogaster cell death machinery. *J Cell Biol* **148:**703–714.

Czauderna F, Fechtner M, Aygun H, Arnold W, Klippel A, Giese K, and Kaufmann J (2003) Functional studies of the PI(3)-kinase signalling pathway employing synthetic and expressed siRNA. *Nucleic Acids Res* **31:**670–682.

Dalmay T, Hamilton A, Rudd S, Angell S, and Baulcombe DC (2000) An RNA-dependent RNA polymerase gene in Arabidopsis is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell* **101:**543–553.

Devroe E and Silver PA (2002) Retrovirus-delivered siRNA. *BMC Biotechnol* **2:**15.

Domeier ME, Morse DP, Knight SW, Portereiko M, Bass BL, and Mango SE (2000) A link between RNA interference and nonsense-mediated decay in Caenorhabditis elegans. *Science (Wash DC)* **289:**1928–1931.

Donze O and Picard D (2002) RNA interference in mammalian cells using siRNAs synthesized with T7 RNA polymerase. *Nucleic Acids Res* **30:**e46.

Duan W, Rangnekar V, and Mattson MP (1999) Par-4 production in synaptic compartments following apoptotic and excitotoxic insults: evidence for a pivotal role in mitochondrial dysfunction and neuronal degeneration. *J Neurochem* **72:**2312–2322.

Duan W, Zhu X, Ladenheim B, Yu QS, Guo Z, Oyler J, Cutler RG, Cadet JL, Greig NH, and Mattson MP (2002) p53 inhibitors preserve dopamine neurons and motor function in experimental parkinsonism. *Ann Neurol* **52:**597–606.

Eddy SR (2001) Non-coding RNA genes and the modern RNA world. *Nat Rev Genet* **2:**919–921.

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, and Tuschl T (2001a) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature (Lond)* **411:**494–498.

Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, and Tuschl T (2001b) Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. *EMBO (Eur Mol Biol Organ) J* **20:**6877–6888.

Eldadah BA and Faden AI (2000) Caspase pathways, neuronal apoptosis and CNS injury. *J Neurotrauma* **17:**811–829.

English JJ, Davenport GF, Elmayan T, Vaucheret H, and Baulcombe DC (1997) Requirement of sense transcription of homology-dependent virus resistance and trans-inactivation. *Plant J* **12:**597–603.

Faergeman NJ and Knudsen J (2002) Acyl-CoA binding protein is an essential protein in mammalian cell lines. *Biochem J* **368:**679–682.

Fagard M, Boutet S, Morel JB, Bellini C, and Vaucheret H (2000) AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi and RNA interference in animals. *Proc Natl Acad Sci USA* **97:**11650–11654.

Fire A (1999) RNA-triggered gene silencing. *Trends Genet* **15:**358–363.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, and Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis Elegans. *Nature (Lond)* **391:**806–811.

Franz DR and Zajtchuk R (2002) Biological terrorism: understanding the threat, preparation and medical response. *Dis Mon* **48:**493–564.

Frischmeyer PA and Dietz HC (1999) Nonsense-mediated mRNA decay in health and disease. *Hum Mol Genet* **8:**1893–1900.

Gan L, Anton KE, Masterson BA, Vincent VA, Ye S, and Gonzalez-Zulueta M (2002) Specific interference with gene expression and gene function mediated by long dsRNA in neural cells. *J Neurosci Methods* **121:**151–157.

Gary DS and Mattson MP (2001) Integrin signaling via the PI3-kinase-Akt pathway increases neuronal resistance to glutamate-induced apoptosis. *J Neurochem* **76:**1485–1496.

Gaudilliere B, Shi Y, and Bonni A (2002) RNA interference reveals a requirement for myocyte enhancer factor 2A in activity-dependent neuronal survival. *J Biol Chem* **277:**46442–46446.

Geng YJ and Libby P (2002) Progression of atheroma: a struggle between death and procreation. *Arterioscler Thromb Vasc Biol* **22:**1370–1380.

Gil J and Esteban M (2000) Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): mechanism of action. *Apoptosis* **5:**107–114.

Gilman CP, Chan SL, Guo Z, Zhu X, Greig NH, and Mattson MP (2003) p53 is present in synapses where it mediates mitochondrial dysfunction and synaptic degeneration in response to DNA damage and oxidative and excitotoxic insults. *Neuromolecular Med* **3:**159–172.

Gitlin L, Karelsky S, and Andino R (2002) Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature (Lond)* **418:**430–434.

Glazner GW, Chan SL, Lu C, and Mattson MP (2000) Caspase-mediated degradation of AMPA receptor subunits: a mechanism for preventing excitotoxic necrosis and ensuring apoptosis. *J Neurosci* **20:**3641–3649.

Greener T, Grant B, Zhang Y, Wu X, Greene LE, Hirsh D, and Eisenberg E (2001) Caenorhabditis elegans auxilin: a J-domain protein essential for clathrin-mediated endocytosis in vivo. *Nat Cell Biol* **3:**215–219.

Grishok A, Tabara H, and Mello CC (2000) Genetic requirements for inheritance of RNAi in C. Elegans. *Science (Wash DC)* **287:**2494–2497.

Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S, Ha I, Baillie DL, Fire A, Ruvkun G, and Mello CC (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. *Cell* **106:**23–34.

Hamilton AJ and Baulcombe DC (1999) A species of small antisense RNA in post-transcriptional gene silencing in plants. *Science (Wash DC)* **286:**950–952.

Hammond SM, Bernstein E, Beach D, and Hannon GJ (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature (Lond)* **404:**293–296.

Hammond SM, Boettcher S, Caudy AA, Kobayashi R, and Hannon GJ (2001) Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science (Wash DC)* **293:**1146–1150.

Harborth J, Elbashir SM, Bechert K, Tuschl T, and Weber K (2001) Identification of essential genes in cultured mammalian cells using small interfering RNAs. *J Cell Sci* **114**:4557–4565.

Hardy J (2001) The genetic causes of neurodegenerative diseases. *J Alzheimers Dis* **3**:109–116.

Higuchi H, Yamashita T, Yoshikawa H, and Tohyama M (2003) Functional inhibition of the p75 receptor using a small interfering RNA. *Biochem Biophys Res Commun* **301**:804–809.

Holtorf H, Schob H, Kunz C, Waldvogel R, and Meins F Jr (1999) Stochastic and nonstochastic post-transcriptional silencing of chitinase and beta-1, 3-glucanase genes involves increased RNA turnover-possible role for ribosome-independent RNA degradation. *Plant Cell* **11**:471–484.

Hutchings NR, Donelson JE, and Hill KL (2002) Trypanin is a cytoskeletal linker protein and is required for cell motility in African trypanosomes. *J Cell Biol* **156**:867–877.

Hutvagner G and Zamore PD (2002) A microRNA in a multiple-turnover RNAi enzyme complex *Science (Wash DC)* **297**:2056–2060.

Iratni R, Yan YT, Chen C, Ding J, Zhang Y, Price SM, Reinberg D, and Shen MM (2002) Inhibition of excess nodal signaling during mouse gastrulation by the transcriptional corepressor DRAP1. *Science (Wash DC)* **298**:1996–1999.

Irie N, Sakai N, Ueyama T, Kajimoto T, Shirai Y, and Saito N (2002) Subtype- and species-specific knockdown of PKC using short interfering RNA. *Biochem Biophys Res Commun* **298**:738–743.

Ishizuka A, Siomi MC, and Siomi H (2002) A Drosophila fragile X protein interacts with components of RNAi and ribosomal proteins. *Genes Dev* **16**:2497–2508.

Jacque JM, Triques K, and Stevenson M (2002) Modulation of HIV-1 replication by RNA interference. *Nature (Lond)* **418**:435–438.

Jansen B and Zangemeister-Wittke U (2002) Antisense therapy for cancer–the time of truth. *Lancet Oncol* **3**:672–683.

Jarad G, Wang B, Khan S, DeVore J, Miao H, Wu K, Nishimura SL, Wible BA, Konieczkowski M, Sedor JR, and Schelling JR (2002) Fas activation induces renal tubular epithelial cell beta 8 integrin expression and function in the absence of apoptosis. *J Biol Chem* **277**:47826–47833.

Jiang M and Milner J (2002) Selective silencing of viral gene expression in HPV-positive human cervical carcinoma cells treated with siRNA, a primer of RNA interference. *Oncogene* **21**:6041–6048.

Kartasheva NN, Contente A, Lenz-Stoppler C, Roth J, and Dobbelstein M (2002) p53 induces the expression of its antagonist p73 Delta N, establishing an autoregulatory feedback loop. *Oncogene* **21**:4715–4727.

Kennerdell JR and Carthew RW (1998) Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 Act in the wingless pathway. *Cell* **95**:1017–1026.

Kennerdell JR and Carthew RW (2000) Heritable gene silencing in Drosophila using double-stranded RNA. *Nat Biotechnol* **18**:896–898.

Ketting RF, Fischer SE, Bernstein E, Sijen T, Hannon GJ, and Plasterk RH (2001) Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. Elegans. *Genes Dev* **15**:2654–2659.

Kim MH, Yuan X, Okumura S, and Ishikawa F (2002) Successful inactivation of endogenous Oct-3/4 and c-mos genes in mouse preimplantation embryos and oocytes using short interfering RNAs. *Biochem Biophys Res Commun* **296**:1372–1377.

Kisielow M, Kleiner S, Nagasawa M, Faisal A, and Nagamine Y (2002) Isoform-specific knockdown and expression of adaptor protein ShcA using small interfering RNA. *Biochem J* **363**:1–5.

Krichevsky AM and Kosik KS (2002) RNAi functions in cultured mammalian neurons. *Proc Natl Acad Sci USA* **99**:11926–11929.

Lee NS, Dohjima T, Bauer G, Li H, Li MJ, Ehsani A, Salvaterra P, and Rossi J (2002a) Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat Biotechnol* **20**:500–505.

Lee SF, Shah S, Li H, Yu C, Han W, and Yu G (2002b) Mammalian APH-1 interacts with presenilin and nicastrin and is required for intramembrane proteolysis of amyloid-beta precursor protein and Notch. *J Biol Chem* **277**:45013–45019.

Lee SS, Lee RY, Fraser AG, Kamath RS, Ahringer J, and Ruvkun G (2002c) A systematic RNAi screen identifies a critical role for mitochondria in C. elegans longevity. *Nat Genet* **33**:40–48.

Leirdal M and Sioud M (2002) Gene silencing in mammalian cells by preformed small RNA duplexes. *Biochem Biophys Res Commun* **295**:744–748.

Lewis DL, Hagstrom JE, Loomis AG, Wolff JA, and Herweijer H (2002) Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nat Genet* **32**:107–108.

Lindenbach BD and Rice CM (2002) RNAi targeting an animal virus: news from the front. *Mol Cell* **9**:925–957.

Liu X and Erikson RL (2002) Activation of Cdc2/cyclin B and inhibition of centrosome amplification in cells depleted of Plk1 by siRNA. *Proc Natl Acad Sci USA* **99**:8672–8676.

Lois C, Hong EJ, Pease S, Brown EJ, and Baltimore D (2002) Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science (Wash DC)* **295**:868–872.

Mailand N, Lukas C, Kaiser BK, Jackson PK, Bartek J, and Lukas J (2002) Deregulated human Cdc14A phosphatase disrupts centrosome separation and chromosome segregation. *Nat Cell Biol* **4**:317–322.

Makimura H, Mizuno TM, Mastaitis JW, Agami R, and Mobbs CV (2002) Reducing hypothalamic AGRP by RNA interference increases metabolic rate and decreases body weight without influencing food intake. *BMC Neurosci* **3**:18.

Marathe R, Anandalakshmi R, Smith TH, Pruss GJ, and Vance VB (2000) RNA viruses as inducers, suppressors and targets of post-transcriptional gene silencing. *Plant Mol Biol* **43**:295–306.

Martinez J, Patkaniowska A, Urlaub H, Luhrmann R, and Tuschl T (2002) Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* **110**:563–574.

Martins LM, Iaccarino I, Tenev T, Gschmeissner S, Totty NF, Lemoine NR, Savopoulos J, Gray CW, Creasy CL, Dingwall C, and Downward J (2002) The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a reaper-like motif. *J Biol Chem* **277**:439–444.

Mattson MP, Keller JN, and Begley JG (1998) Evidence for synaptic apoptosis. *Exp Neurol* **153**:35–48.

Mattson MP (2000) Apoptosis in neurodegenerative disorders. *Nat Rev Mol Cell Biol* **1**:120–129.

Mattson MP (2003) Excitotoxic and excitoprotective mechanisms: abundant targets for the prevention and treatment of neurodegenerative disorders. *Neuromolecular Med* **3**:65–94.

Mattson MP and Camandola S (2001) NF-kappaB in neuronal plasticity and neurodegenerative disorders. *J Clin Investig* **107**:247–254.

Mattson MP, Culmsee C, and Yu ZF (2000) Apoptotic and antiapoptotic mechanisms in stroke. *Cell Tissue Res* **301**:173–187.

McCaffrey AP, Meuse L, Pham TT, Conklin DS, Hannon GJ, and Kay MA (2002) RNA interference in adult mice. *Nature (Lond)* **418**:38–39.

McManus MT, Petersen CP, Haines BB, Chen J, and Sharp PA (2002) Gene silencing using micro-RNA designed hairpins. *RNA* **8**:842–850.

Mendell JT, Rhys CM, and Dietz HC (2002) Separable roles for rent1/hUpf1 in altered splicing and decay of nonsense transcripts. *Science (Wash DC)* **298**:419–422.

Mette MF, Aufsatz W, van der Winden J, Matzke MA, and Matzke AJ (2000) Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *EMBO (Eur Mol Biol Organ) J* **19**:5194–5201.

Mise-Omata S, Montagne B, Deckert M, Wienands J, and Acuto O (2003) Mammalian actin binding protein 1 is essential for endocytosis but not lamellipodia formation: functional analysis by RNA interference. *Biochem Biophys Res Commun* **301**:704–710.

Miyagishi M and Taira K (2002) U6 promoter-driven siRNAs with four uridine 3′ overhangs efficiently suppress targeted gene expression in mammalian cells. *Nat Biotechnol* **20**:497–500.

Mollinari C, Kleman JP, Jiang W, Schoehn G, Hunter T, and Margolis RL (2002) PRC1 is a microtubule binding and bundling protein essential to maintain the mitotic spindle midzone. *J Cell Biol* **157**:1175–1186.

Montgomery MK, Xu S, and Fire A (1998) RNA as a target of double-stranded RNA-mediated genetic interference in Caenorhabditis Elegans. *Proc Natl Acad Sci USA* **95**:15502–15507.

Mourrain P, Beclin C, Elmayan T, Feuerbach F, Godon C, Morel JB, Jouette D, Lacombe AM, Nikic S, Picault N, Remoue K, Sanial M, Vo TA, Vaucheret H (2000) Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* **101**:533–542.

Nagashima K, Endo A, Ogita H, Kawana A, Yamagishi A, Kitabatake A, Matsuda M, and Mochizuki N (2002) Adaptor protein Crk is required for ephrin-B1-induced membrane ruffling and focal complex assembly of human aortic endothelial cells. *Mol Biol Cell* **13**:4231–4242.

Naldini L (1998) Lentiviruses as gene transfer agents for delivery to non-dividing cells. *Curr Opin Biotechnol* **9**:457–463.

Nam NH and Parang K (2003) Current targets for anticancer drug discovery. *Curr Drug Targets* **4**:159–179.

Napoli C, Lemieux C, and Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *Plant Cell* **2**:279–289.

Nicholson RH and Nicholson AW (2002) Molecular characterization of a mouse cDNA encoding Dicer, a ribonuclease III ortholog involved in RNA interference. *Mamm Genome* **13**:67–73.

Novina CD, Murray MF, Dykxhoorn DM, Beresford PJ, Riess J, Lee SK, Collman RG, Lieberman J, Shankar P, and Sharp PA (2002) siRNA-directed inhibition of HIV-1 infection. *Nat Med* **8**:681–686.

Nykanen A, Haley B, and Zamore PD (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* **107**:309–321.

Ohta T, Essner R, Ryu JH, Palazzo RE, Uetake Y, and Kuriyama R (2002) Characterization of Cep135, a novel coiled-coil centrosomal protein involved in microtubule organization in mammalian cells. *J Cell Biol* **156**:87–99.

Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, and Conklin DS (2002a) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev* **16**:948–958.

Paddison PJ, Caudy AA, and Hannon GJ (2002b) Stable suppression of gene expression by RNAi in mammalian cells. *Proc Natl Acad Sci USA* **99**:1443–1448.

Page MF, Carr B, Anders KR, Grimson A, and Anderson P (1999) SMG-2 is a phosphorylated protein required for mRNA surveillance in Caenorhabditis elegans and related to Upf1p of yeast. *Mol Cell Biol* **19**:5943–5951.

Paul CP, Good PD, Winer I, and Engelke DR (2002) Effective expression of small interfering RNA in human cells. *Nat Biotechnol* **20**:505–508.

Peng Y, Zhang Q, Nagasawa H, Okayasu R, Liber HL, and Bedford JS (2002) Silencing expression of the catalytic subunit of DNA-dependent protein kinase by small interfering RNA sensitizes human cells for radiation-induced chromosome damage, cell killing and mutation. *Cancer Res* **62**:6400–6404.

Pfeifer A, Ikawa M, Dayn Y, and Verma IM (2002) Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. *Proc Natl Acad Sci USA* **99**:140–145.

Poinat P, De Arcangelis A, Sookhareea S, Zhu X, Hedgecock EM, Labouesse M, and Georges-Labouesse E (2002) A conserved interaction between beta1 integrin/PAT-3 and Nck-interacting kinase/MIG-15 that mediates commissural axon navigation in C. elegans. *Curr Biol* **12**:622–631.

Porter LA, Dellinger RW, Tynan JA, Barnes EA, Kong M, Lenormand JL, and Donoghue DJ (2002) Human Speedy: a novel cell cycle regulator that enhances proliferation through activation of Cdk2. *J Cell Biol* **157**:357–366.

Prasanth SG, Prasanth KV, and Stillman B (2002) Orc6 involved in DNA replication, chromosome segregation and cytokinesis. *Science (Wash DC)* **297**:1026–1031.

Pruss G, Ge X, Shi XM, Carrnington JC, and Vance VB (1997) Plant viral synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. *Plant Cell* **9**:1749–1759.

Quinn LM, Dorstyn L, Mills K, Colussi PA, Chen P, Coombe M, Abrams J, Kumar S, and Richardson H (2000) An essential role for the caspase dronc in developmentally programmed cell death in Drosophila. *J Biol Chem* **275:**40416–40424.

Randall G, Grakoui A, and Rice CM (2003) Clearance of replicating hepatitis C virus replicon RNAs in cell culture by small interfering RNAs. *Proc Natl Acad Sci USA* **100:**235–240.

Ratcliff F, Harrison BD, and Baulcombe DC (1997) A similarity between viral defense and gene silencing in plants. *Science (Wash DC)* **276:**1558–1560.

Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, Kopinja J, Zhang M, McManus MT, Gertler FB, Scott ML, and Van Parijs L (2003) A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat Genet* **33:**401–406.

Rubinsztein DC (2002) Lessons from animal models of Huntington's disease. *Trends Genet* **18:**202–209.

Ruiz-Echevarria MJ, Czaplinski K, and Peltz SW (1996) Making sense of nonsense in yeast. *Trends Biochem Sci* **21:**433–438.

Salisbury JL, Suino KM, Busby R, and Springett M (2002) Centrin-2 is required for centriole duplication in mammalian cells. *Curr Biol* **12:**1287–1292.

Siegmund D, Hadwiger P, Pfizenmaier K, Vornlocher HP, and Wajant H (2002) Selective inhibition of FLICE-like inhibitory protein expression with small interfering RNA oligonucleotides is sufficient to sensitize tumor cells for TRAIL-induced apoptosis. *Mol Med* **8:**725–732.

Smalheiser NR, Manev H, and Costa E (2001) RNAi and brain function: was McConnell on the right track? *Trends Neurosci* **24:**216–218.

Smardon A, Spoerke JM, Stacey SC, Klein ME, Mackin N, and Maine EM (2000) EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in C. elegans. *Curr Biol* **10:**169–178.

Smith GM and Romero MI (1999) Adenoviral-mediated gene transfer to enhance neuronal survival, growth and regeneration. *J Neurosci Res* **55:**147–157.

Song E, Lee SK, Wang J, Ince N, Ouyang N, Min J, Chen J, Shankar P, and Lieberman J (2003) RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat Med* **9:**347–351.

Stucke VM, Sillje HH, Arnaud L, and Nigg EA (2002) Human Mps1 kinase is required for the spindle assembly checkpoint but not for centrosome duplication. *EMBO (Eur Mol Biol Organ) J* **21:**1723–1732.

Sui G, Soohoo C, Affar el B, Gay F, Shi Y, Forrester WC, Shi Y (2002) A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc Natl Acad Sci USA* **99:**5515–5520.

Surabhi RM and Gaynor RB (2002) RNA interference directed against viral and cellular targets inhibits human immunodeficiency virus type 1 replication. *J Virol* **76:**12963–12973.

Svoboda P, Stein P, and Schultz RM (2001) RNAi in mouse oocytes and preimplantation embryos: effectiveness of hairpin dsRNA. *Biochem Biophys Res Commun* **287:**1099–1104.

Tabara H, Grishok A, and Mello CC (1998) RNAi in C. elegans: soaking in the genome sequence. *Science (Wash DC)* **282:**430–431.

Tabara H, Sarkissian M, Kelly WG, Fleenor J, Grishok A, Timmons L, Fire A, and Mello CC (1999) The Rde-1 gene, RNA interference and transposon silencing in C. Elegans. *Cell* **99:**123–132.

Tabara H, Yigit E, Siomi H, and Mello CC (2002) The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1 and a DExH-box helicase to direct RNAi in C. elegans. *Cell* **109:**861–871.

Tan YT, Tillett DJ, and McKay IA (2000) Molecular strategies for overcoming antibiotic resistance in bacteria. *Mol Med Today* **6:**309–314.

Tavernarakis N, Wang SL, Dorovkov M, Ryazanov A, and Driscoll M (2000) Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. *Nat Genet* **24:**180–183.

Timmons L and Fire A (1998) Specific interference by ingested dsRNA. *Nature (Lond)* **395:**854.

Tiscornia G, Singer O, Ikawa M, and Verma IM (2003) A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. *Proc Natl Acad Sci USA* **100:**1844–1848.

Tsuneoka M, Koda Y, Soejima M, Teye K, and Kimura H (2002) A novel myc target gene, mina53, that is involved in cell proliferation. *J Biol Chem* **277:**35450–35459.

Tuschl T, Zamore PD, Lehmann R, Bartel DP, and Sharp PA (1999) Targeted mRNA degradation by double-stranded RNA in vitro. *Genes Dev* **13:**3191–3197.

Ukomadu C and Dutta A (2003) Inhibition of cdk2 activating phosphorylation by mevastatin. *J Biol Chem* **278:**4840–4846.

Van Blokland R, Van Der Geest N, Mol JMN, and Kooter JM (1994) Transgene-mediated expression of chalcone synthase expression in Petunia hybrida results from an increase in RNA turnover. *Plant J* **6:**861–877.

Volpe TA, Kidner C, Hall IM, Teng G, Grewal SI, and Martienssen RA (2002) Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science (Wash DC)* **297:**1833–1837.

Walters DK and Jelinek DF (2002) The effectiveness of double-stranded short inhibitory RNAs (siRNAs) may depend on the method of transfection. *Antisense Nucleic Acid Drug Dev* **12:**411–418.

Wang X, Yang C, Chai J, Shi Y, and Xue D (2002) Mechanisms of AIF-mediated apoptotic DNA degradation in Caenorhabditis elegans. *Science (Wash DC)* **298:**1587–1592.

Wang Z, Morris JC, Drew ME, and Englund PT (2000) Inhibition of Trypanosoma brucei gene expression by RNA interference using an integratable vector with opposing T7 promoters. *J Biol Chem* **275:**40174–40179.

Weil D, Garcon L, Harper M, Dumenil D, Dautry F, and Kress M (2002) Targeting the kinesin Eg5 to monitor siRNA transfection in mammalian cells. *Biotechniques* **33:**1244–1248.

Wianny F and Zernicka-Goetz M (2000) Specific interference with gene function by double-stranded RNA in early mouse development. *Nat Cell Biol* **2:**70–75.

Wilda M, Fuchs U, Wossmann W, and Borkgardt A (2002) Killing of leukemic cells with a BCR/ABL fusion gene by RNA interference (RNAi). *Oncogene* **21:**5716–5724.

Wilson JA, Jayasena S, Khvorova A, Sabatinos S, Rodrigue-Gervais IG, Arya S, Sarangi F, Harris-Brandts M, Beaulieu S, and Richardson CD (2003) RNA interference blocks gene expression and RNA synthesis from hepatitis C replicons propagated in human liver cells. *Proc Natl Acad Sci USA* **100:**2783–2788.

Winston WM, Molodowitch C, and Hunter CP (2002) Systemic RNAi in C. elegans requires the putative transmembrane protein SID-1. *Science (Wash DC)* **295:**2456–2459.

Wojcik C and DeMartino GN (2002) Analysis of Drosophila 26 S proteasome using RNA interference. *J Biol Chem* **277:**6188–6197.

Wong SK and Lazinski DW (2002) Replicating hepatitis delta virus RNA is edited in the nucleus by the small form of ADAR1. *Proc Natl Acad Sci USA* **99:**15118–15123.

Xia H, Mao Q, Paulson HL, and Davidson BL (2002) siRNA-mediated gene silencing in vitro and in vivo. *Nat Biotechnol* **20:**1006–1010.

Xu K, Tavernarakis N, and Driscoll M (2001) Necrotic cell death in C. elegans requires the function of calreticulin and regulators of Ca(2+) release from the endoplasmic reticulum. *Neuron* **31:**957–971.

Xu Z, Williams CJ, Kopf GS, and Schultz RM (2003) Maturation-associated increase in IP(3) receptor type 1: role in conferring increased IP(3) sensitivity and Ca(2+) oscillatory behavior in mouse eggs. *Dev Biol* **254:**163–171.

Yang S, Tutton S, Pierce E, and Yoon K (2001) Specific double-stranded RNA interference in undifferentiated mouse embryonic stem cells. *Mol Cell Biol* **21:**7807–7816.

Ye F and Signer ER (1996) RIGS (repeat-induced gene silencing) in Arabidopsis is transcriptional and alters chromatin configuration. *Proc Natl Acad Sci USA* **93:**10881–10886.

Yu JY, DeRuiter SL, and Turner DL (2002) RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc Natl Acad Sci USA* **99:**6047–6052.

Zamore PD, Tuschl T, Sharp PA, and Bartel DP (2000) RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101:**25–33.

Zhang D, Li F, Weidner D, Mnjoyan ZH, and Fujise K (2002a) Physical and functional interaction between myeloid cell leukemia 1 protein (MCL1) and Fortilin. The potential role of MCL1 as a fortilin chaperone. *J Biol Chem* **277:**37430–37438.

Zhang H, Kolb FA, Brondani V, Billy E, and Filipowicz W (2002b) Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. *EMBO (Eur Mol Biol Organ) J* **21:**5875–5885.

Zhao ZQ and Vinten-Johansen J (2002) Myocardial apoptosis and ischemic preconditioning. *Cardiovasc Res* **55:**438–455.

Zhu X, Yu QS, Cutler RG, Culmsee CW, Holloway HW, Lahiri DK, Mattson MP, and Greig NH (2002) Novel p53 inactivators with neuroprotective action: syntheses and pharmacological evaluation of 2-imino-2,3,4,5,6,7-hexahydrobenzothiazole and 2-imino-2,3,4,5,6,7-hexahydrobenzoxazole derivatives. *J Med Chem* **45:**5090–5097.

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RNA Interference: Biology, Mechanism, and Applications

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INTRODUCTION

RNA silencing is a novel gene regulatory mechanism that limits the transcript level by either suppressing transcription (transcriptional gene silencing [TGS]) or by activating a sequence-specific RNA degradation process (posttranscriptional gene silencing [PTGS]/RNA interference [RNAi]). Although there is a mechanistic connection between TGS and PTGS, TGS is an emerging field while PTGS is undergoing an explosion in its information content. Here, we have limited our discussion to PTGS/RNAi-related phenomena.

Pioneering observations on PTGS/RNAi were reported in plants, but later on RNAi-related events were described in almost all eukaryotic organisms, including protozoa, flies, nematodes, insects, parasites, and mouse and human cell lines, as shown in Table 1. Three phenotypically different but mechanistically similar forms of RNAi, cosuppression or PTGS in

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TABLE 1. Eukaryotic organisms exhibiting RNAi-related phenomena

Kingdom	Species	Stage tested	Delivery method	Reference(s)
Protozoans	*Trypanosoma brucei*	Procyclic forms	Transfection	52
	Plasmodium falciparum	Blood stage	Electroporation and soaking	143, 150
	Toxoplasma gondii	Mature forms in fibroblast	Transfection	4
	Paramecium	Mature form	Transfection and feeding	14
	Leishmania donovanii		Tried but not working	183
Invertebrates	*Caenorhabditis elegans*	Larval stage and adult stage	Transfection, feeding bacteria carrying dsRNA, soaking	26, 31
	Caenorhabditis briggsae	Adult	Injection	79
	Brugia malayi (filarial worm)	Adult worm	Soaking	1
	Schistosoma mansoni	Sporocysts	Soaking	23
	Hydra	Adult	Delivered by micropipette	49
	Planaria	Adult	Soaking	49
	Lymnea stagnalis (snail)	Adult	Injection	122
	Drosophila melanogaster	Cell lines, adult, embryo	Injection for adult and embryonic stages, soaking and transfection for cell lines	96, 114, 155
	Cyclorrphan (fly)	Early embryonic stages	Injection	200
	Milkweed bug	Early embryonic stages	Injection	102
	Beetle	Early embryonic stages	Injection	27
	Cockroach	Larval stage	Injection	146
	Spodoptera frugiperda	Adult and cell line	Injection and soaking	176, 215
Vertebrates	Zebra fish	Embryo	Microinjection	224
	Xenopus laevis	Embryo	Injection	162
	Mice	Prenatal, embryonic stages, and adult	Injection	31, 229
	Humans	Human cell lines	Transfection	42
Plants	Monocots/dicots	Plant	Particle bombardment with siRNA/transgenics	88
Fungi	*Neurospora crassa*	Filamentous fungi	Transfection	51
	Schizosaccharomyces pombe	Filamentous fungi	Transgene	178
	Dictyostelium discoideum		Transgene	147
Algae	*Chlamydomonas reinhardtii*		Transfection	231

plants, quelling in fungi, and RNAi in the animal kingdom, have been described. More recently, micro-RNA formation, heterochromatinization, etc., have been revealed as other facets of naturally occurring RNAi processes of eukaryotic cells.

During the occurrence of RNAi/PTGS, double-stranded RNA (dsRNA) molecules, which cleave the inducer molecules into smaller pieces first (16) and eventually destroy the cellular or viral cognate mRNA molecules (called the target) (17) act as inducers or activators of this process. As a result, the target mRNAs cannot accumulate in the cytosol, although they remain detectable by nuclear run-on assays (73). In certain instances, the DNA expressing the target mRNA also undergoes methylation as a by-product of the degradation process (226).

The natural functions of RNAi and its related processes seem to be protection of the genome against invasion by mobile genetic elements such as viruses and transposons as well as orchestrated functioning of the developmental programs of eukaryotic organisms. There are several excellent recent reviews which deal with different aspects of RNAi separately (95, 191). Here, we have put together the various aspects of the RNAi process known to date, identified the mechanistic similarities and differences operating in various forms of eukaryotic life, and focused on the experimental results that have led to conceptual advancements in this field.

UNRAVELING RNA SILENCING

In order to understand the process of homology-dependent RNA silencing, it would be prudent to overview the process itself and describe its important features. In the later part of this review, the genetics, biochemistry, and potential therapeutic applications of the process will be dealt with.

PTGS in Plants

In plants, the RNA silencing story unfolded serendipitously during a search for transgenic petunia flowers that were expected to be more purple. In 1990, R. Jorgensen's laboratory wanted to upregulate the activity of a gene for chalcone synthase (*chsA*), an enzyme involved in the production of anthocyanin pigments. Surprisingly, some of the transgenic petunia plants harboring the *chsA* coding region under the control of a 35S promoter lost both endogene and transgene chalcone synthase activity, and thus many of the flowers were variegated or developed white sectors (163). The loss of cytosolic *chsA* mRNA was not associated with reduced transcription, as demonstrated by run-on transcription tests in isolated nuclei (216). Jorgensen coined the term cosuppression to describe the loss of mRNAs of both the endo- and the transgene.

Around the same time, two other laboratories (105, 217) also reported that introduction of the transcribing-sense transgenes could downregulate the expression of homologous endogenous genes. Subsequently, many similar events of cosuppression were reported in the literature. All cases of cosuppression resulted in the degradation of endogene and transgene RNAs after nuclear transcription had occurred (120). Since posttranscriptional RNA degradation was observed in a wide range of transgenes expressing the plant, bacterial, or viral sequences, it was rechristened posttranscriptional gene silencing (PTGS). PTGS could be initiated not only by sense transgenes but also by antisense transgenes, and biochemical evidence suggests that similar mechanisms might operate in both cases (81). It is worthwhile to point out that although the cosuppression phenomenon was originally observed in plants, it is not restricted to plants and has also been demonstrated in metazoans and mammals (98).

In keeping with the times, the observed alterations in the PTGS-related phenotypes were attributed to multiple-site integrations, aberrant RNA formations, repeat structures of the transgenes, etc. Later on, it became clear that the expression of the transgene led to the formation of dsRNA, which, in turn, initiated PTGS. For example, in the case of cosuppressed petunia plants, *chsA* mRNA formed a partial duplex, since there are regions of self-complementarity located between *chsA* 3′ coding region and its 3′ untranslated region (154). This was revealed by DNA sequence analysis and experimental detection of in vitro-transcribed, RNase-resistant duplex *chsA* RNA. In an independent study, a p35S-ACC (1-aminocyclopropane-1-carboxylate [ACC] oxidase) sense transgene carrying a small inverted repeat in the 5′ untranslated region was introduced into tomato to test the role of dsRNA structure as an inducer of PTGS. Cosuppression of the endogenous *acc* gene occurred at a higher frequency in these plants than in those harboring only the p35S-ACC sense transgene without the inverted repeat (93).

Reports from several laboratories in the past few years have established that the loss in steady-state accumulation of the target mRNA is almost total if the designed transgene construct of the transgenic plant produces the nuclear transcript in the duplex conformation. Very recently it was reported that the expression of self-cRNA of plum pox virus under the control of *rolC* promoter caused degradation of transgenic viral RNA and as a result, the systemic disease resistance to challenge inoculum of plum pox virus occurred with a high frequency in transgenic *Nicotiana benthamiana* (170). This evidence points out that the production of dsRNA is required to initiate PTGS in plants. Based on this, plants carrying strongly transcribing transgenes in both the sense and antisense orientations are currently being produced that show strong PTGS features. These transgenic plants can silence endogene, invading viral RNA, or unwanted foreign genes in a sequence-specific and heritable manner.

Generally, the sense and antisense components of the above-mentioned transgenes are separated only by an intron to increase the efficacy of PTGS (43, 198). For example, *Arabidopsis thaliana* and *Lycopersicon esculentum* (tomato) plants were transformed with a transgene construct designed to generate self-complementary *iaaM* and *ipt* transcripts. *iaaM* and *ipt* are oncogenes of agrobacteria that are responsible for crown gall formation in infected plants. The transgenic lines retained susceptibility to *Agrobacterium* transformation but were highly refractory to tumorigenesis, providing functional resistance to crown gall disease by posttranscriptional degradation of the *iaaM* and *ipt* transcripts (72).

Quelling and RNAi

While reports of PTGS in plants were piling up, homology-dependent gene silencing phenomena were also observed independently in fungal systems. These events were called quelling. Quelling came to light during attempts to boost the production of an orange pigment made by the gene *al1* of the fungus *Neurospora crassa* (50). An *N. crassa* strain containing a wild-type $al1^+$ gene (orange phenotype) was transformed with a plasmid containing a 1,500-bp fragment of the coding sequence of the *al1* gene. A few transformants were stably quelled and showed albino phenotypes. In the *al1*-quelled strain, the level of unspliced *al1* mRNA was similar to that of the wild-type strain, whereas the native *al1* mRNA was highly reduced, indicating that quelling and not the rate of transcription affected the level of mature mRNA in a homology-dependent manner.

The phenomenon of RNAi first came into the limelight following the discovery by Fire et al. (78), who unequivocally demonstrated the biochemical nature of inducers in gene silencing by introducing purified dsRNA directly into the body of *Caenorhabditis elegans*. The investigators injected dsRNA corresponding to a 742-nucleotide segment of *unc22* into either the gonad or body cavity region of an adult nematode. *unc22* encodes an abundant but nonessential myofilament protein, and the decrease in *unc22* activity is supposed to produce an increasingly severe twitching phenotype. The injected animal showed weak twitching, whereas the progeny individuals were strong twitchers. The investigators showed that similar loss-of-function individuals could also be generated with dsRNAs corresponding to four other nematode genes. The phenotypes produced by interference by various dsRNAs were extremely specific.

This experiment paved the way for easy production of null mutants, and the process of silencing a functional gene by exogenous application of dsRNA was termed RNA interference (RNAi). RNAi in *C. elegans* was also initiated simply by soaking the worms in a solution containing dsRNAs or by feeding the worms *Escherichia coli* organisms that expressed the dsRNAs (209). This is a very potent method, requiring only catalytic amounts of dsRNA per cell to silence gene expression. The silencing spread not only from the gut of the worm to the remainder of the body, but also through the germ line to several generations. These phenomena of RNAi have also been demonstrated to occur in *Drosophila melanogaster* and many other invertebrates and vertebrates.

Insights from Virus-Infected Plants (Virus-Induced Gene Silencing)

Besides the processes mentioned above, homology-driven RNA degradation also occurs during the growth of viral genomes in infected plants (73). Viruses can be either the source, the target, or both the source and the target of silencing. PTGS

mediated by viruses can occur with RNA viruses, which replicate in the cytoplasm, and also with DNA viruses, which replicate in the nucleus (71). As early as in the 1920s, it was known that plants could be protected from a severe virus by prior infection with a mild strain of a closely related virus. Although the mechanism of such cross protection in plants remained unknown for a long time, such phenomena could be explained partly in terms of PTGS that could be induced by the mild strain and targeted later against the virulent viral genome. It was also found that transforming plants with virus-derived transgenes gave protection against the challenge viruses even when no transgene protein was produced (132).

Analyses of these virus-resistant plants revealed that the transgenes were highly transcribed in the nucleus, whereas the steady-state level of cytoplasmic mRNA was very low. Further analysis suggested that some of the transgenic mRNA molecules assumed the conformation of dsRNA, which triggered sequence-specific degradation of self and other homologous or cRNA sequences in the cytoplasm. Thus, in the virus-resistant lines, not only the transgene mRNAs but also the mRNA from the homologous endogenous gene and the invading viral RNA (with homology to the transgene) were degraded.

Another form of virus-induced gene silencing is the phenomenon of viral recovery itself. When *Brassica napus* was inoculated with cauliflower mosaic virus (a DNA virus), lesions at the site of virus entry were visible 5 to 7 days postinoculation. Symptoms of systemic infections were apparent by 10 to 14 days postinoculation. Symptoms were most prominent at 30 to 40 days postinoculation and declined thereafter (i.e., the plants recovered), with the newly emergent leaves remaining asymptomatic at 50 days postinoculation (5).

Figure 1 diagrammatically illustrates the systemic spread of RNAi in plants. Such recovery occurred by a PTGS-like mechanism because 19S and 35S RNAs encoded by the cauliflower mosaic virus were degraded while cauliflower mosaic virus DNA was still replicating in the nucleus. Induction of PTGS was visualized if the cauliflower mosaic virus infection and subsequent recovery were followed up in a transgenic *B. napus* expressing a p35S-GUS (β-glucuronidase) transgene. At the site of inoculation, GUS silencing associated with local lesions was first observed 7 days postinoculation. GUS silencing eventually spread systemically, and the GUS activity of the entire plant was suppressed by 50 days postinoculation. In this particular example, cauliflower mosaic virus acted as the inducer of PTGS for the transgenes sharing homology with the virus within the transcribed region. However, the virus itself was also the target of the induced PTGS, since 19S and 35S RNAs were found degraded.

A similar example of virus-induced gene silencing was found when *Nicotiana clevelandii* was infected with an RNA nepovirus, tomato black ring virus (179). RNA viruses make abundant dsRNA during intracellular replication of their genomes and thus elicit cellular PTGS degradative activity. Virus-induced gene silencing also occurs with viruses that do not undergo recovery. When a DNA geminivirus, tomato golden mosaic virus (TGMV), infected *N. benthamiana*, a high level of viral DNA replication in the nucleus and accumulation of viral RNA in the cytoplasm occurred. An infection by a recombinant TGMV carrying the coding sequence of the sulfur (*su*) gene of the host plant in either the sense or antisense orientation led to the bleaching of leaves due to PTGS of the endogenous *su* gene, but the DNA of the recombinant did not fail to replicate (117). Here, TGMV acted as an inducer of PTGS but was not itself a target of PTGS. Thus, plant viruses elicit PTGS but sometimes can escape the degradative PTGS activity.

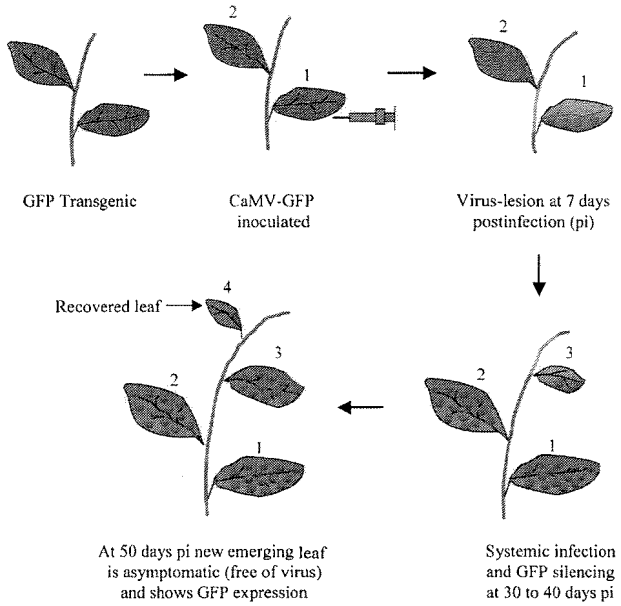

FIG. 1. Schematic illustration of systemic viral spread as well as RNAi and subsequent viral recovery in plants. Green and red indicate the presence and loss of GFP fluorescence, respectively, and orange denotes the presence of both colors. The red dots on leaves show viral lesions. The bold arrows indicate the stages of plant growth, and the leaves are numbered accordingly. An arrow with a thin line shows a newly emerged leaf recovered from viral attack.

Based on the principles of virus-induced gene silencing, vectors designed with the genome sequence of RNA viruses tobacco mosaic virus, potato virus X, and tobacco rattle virus are being widely used to knock down the expression of host genes. The characteristics of many plant genes were revealed by observing the loss-of-function-related phenotypic changes when the recombinant vectors incorporating the concerned host genes were introduced into plants (136). Of these vectors, the TRV-based are more promising because these are capable of inducing-meristematic gene silencing, which has not been possible to achieve with other RNA virus-based vectors. Meristematic gene silencing employing TGMV vectors has also been reported (173). Thus, virus-induced gene silencing-based techniques are extremely useful for studies related to functional genomics in plants.

IMPORTANT FEATURES OF RNA SILENCING

Independently of one another, investigations on diverse organisms, labeled variously as PTGS in plants, RNAi in animals, quelling in fungi, and virus-induced gene silencing, have converged on a universal paradigm of gene regulation. The critical common components of the paradigm are that (i) the inducer is the dsRNA, (ii) the target RNA is degraded in a homology-dependent fashion, and, as we will see later, (iii) the degrada-

tive machinery requires a set of proteins which are similar in structure and function across most organisms. In most of these processes, certain invariant features are observed, including the formation of small interfering RNA (siRNA) and the organism-specific systemic transmission of silencing from its site of initiation.

siRNA

The key insight in the process of PTGS was provided from the experiments of Baulcombe and Hamilton (92), who identified the product of RNA degradation as a small RNA species (siRNA) of ≈25 nucleotides of both sense and antisense polarity. siRNAs are formed and accumulate as double-stranded RNA molecules of defined chemical structures, as mentioned later. siRNAs were detected first in plants undergoing either cosuppression or virus-induced gene silencing and were not detectable in control plants that were not silenced. siRNAs were subsequently discovered in *Drosophila* tissue culture cells in which RNAi was induced by introducing >500-nucleotide-long exogenous dsRNA (96), in *Drosophila* embryo extracts that were carrying out RNAi in vitro (240), and also in *Drosophila* embryos that were injected with dsRNA (236). Thus, the generation of siRNA (21 to 25 nucleotides) turned out to be the signature of any homology-dependent RNA-silencing event.

The siRNAs resemble breakdown products of an *E. coli* RNase III-like digestion (13). In particular, each strand of siRNA has 5′-phosphate and 3′-hydroxyl termini and 2- to 3-nucleotide 3′ overhangs. Interestingly, in vitro-synthesized siRNAs can, in turn, induce specific RNA degradation when added exogenously to *Drosophila* cell extracts (69). Specific inhibition of gene expression by these siRNAs has also been observed in many invertebrate and some vertebrate systems (67). Recently, Schwarz et al. (189) provided direct biochemical evidence that the siRNAs could act as guide RNAs for cognate mRNA degradation.

Amplification and Systemic Transmission

Besides the formation of siRNAs, another intriguing characteristic of homology-dependent gene silencing is that the inducer dsRNA molecules do not act stoichiometrically. It was estimated that only two molecules of dsRNA per cell were able to induce RNAi of an abundantly expressed *C. elegans* gene such as *unc22*. In another report, injection of dsRNA into the intestine of a *C. elegans* hermaphrodite generated RNAi, which could be stably inherited to the F_2 generation. These two findings led to the proposal that RNAi signals could be systemic and amplifiable in nature (78). The similar systemic effects of RNAi have also been demonstrated in the planarian *Schmidtea mediterranea* and the cnidarian *Hydra magnipapillata* (140).

Similar evidence is also available for plant PTGS. The new tissues growing from a GUS-expressing scion grafted onto a GUS-silenced rootstock show progressive silencing of GUS expression (168). The silencing signal seems to spread by a nonmetabolic, gene-specific diffusible signal, which travels both between cells, through plasmadesmata, and long distances via the phloem (75). In the case of virus-induced gene silencing, the systemic character has also been revealed (185). To account for the gene specificity of a systemic signal, it has been proposed that the signal could be an RNA molecule (228). However, such processes are not universal, as these are not found in flies and mammals.

COMPONENTS OF GENE SILENCING

Both genetic and biochemical approaches have been undertaken to understand the basis of silencing. Genetic screens were carried out in the fungus *Neurospora crassa*, the alga *Chlamydomonas reinhardtii*, the nematode *Caenorhabditis elegans*, and the plant *A. thaliana* to search for mutants defective in quelling, RNA interference, or PTGS. Analyses of these mutants led to the identification of host-encoded proteins involved in gene silencing and also revealed that a number of essential enzymes or factors are common to these processes. Some of the components identified serve as initiators, while others serve as effectors, amplifiers, and transmitters of the gene silencing process. In the years to come, many other components as well as their interrelations will be revealed. Here, we outline what is known so far.

Dicer

RNase III family members are among the few nucleases that show specificity for dsRNAs (164) and cleave them with 3′ overhangs of 2 to 3 nucleotides and 5′-phosphate and 3′-hydroxyl termini (69). Bernstein et al. (17) identified an RNase III-like enzyme in *Drosophila* extract which was shown to have the ability to produce fragments of 22 nucleotides, similar to the size produced during RNAi. These authors showed that this enzyme is involved in the initiation of RNAi. Owing to its ability to digest dsRNA into uniformly sized small RNAs (siRNA), this enzyme was named Dicer (DCR). These nucleases are evolutionarily conserved in worms, flies, fungi, plants, and mammals. Dicer has four distinct domains: an amino-terminal helicase domain, dual RNase III motifs, a dsRNA binding domain, and a PAZ domain (a 110-amino-acid domain present in proteins like Piwi, Argo, and Zwille/Pinhead), which it shares with the RDE1/QDE2/Argonaute family of proteins that has been genetically linked to RNAi by independent studies (34, 203). Cleavage by Dicer is thought to be catalyzed by its tandem RNase III domains. Some DCR proteins, including the one from *D. melanogaster*, contain an ATP-binding motif along with the DEAD box RNA helicase domain.

The predicted *C. elegans* Dicer homologue, K12H4.8, was referred as DCR1 because it was demonstrated to be the functional ortholog of the *Drosophila* Dicer protein (173). The 8,165-bp DCR1 protein has a domain structure similar to that of the *Drosophila* Dicer protein. *dcr1* mutants of *C. elegans* showed defects in RNAi of germ line-expressed genes but no effect on the RNAi response of somatic genes. These mutants were found to be sterile, suggesting the important role of this gene in germ line development apart from RNAi (119). CAF1 has been identified as a Dicer homologue in *A. thaliana*, but it is not involved in PTGS activity. The structure of CAF1 shows the presence of the four distinct domains that were identified in the *Drosophila* Dicer protein (17, 36, 108). Dicer homologues from many different sources have been identified; some

recombinant Dicers have also been examined in vitro, and phylogenetic analysis of the known Dicer-like proteins indicates a common ancestry of these proteins (83).

Complete digestion by RNase III enzyme results in dsRNA fragments of 12 to 15 bp, half the size of siRNAs (235). The RNase III enzyme acts as a dimer and thus digests dsRNA with the help of two compound catalytic centers, whereas each monomer of the Dicer enzyme possesses two catalytic domains, with one of them deviating from the consensus catalytic sequences.

Recently, the crystal structure of the RNase III catalytic domain was solved, and this led to the model for generation of 23- to 28-mer diced siRNA products (20). In this model, the dimeric Dicer folds on the dsRNA substrate to produce four compound catalytic sites so that the two terminal sites having the maximum homology with the consensus RNase III catalytic sequence remain active, while the other two internal sites bearing partial homology lose functional significance. Thus, the diced products appear as the limit digests of the RNase III enzymes and are double the size of the normal 12- to 15-mer fragments. Such a model also predicts that certain changes in Dicer structure might modify the spacing between the two active terminal sites and thus generate siRNAs of variable sizes bearing species-specific imprints (98). Clearly, the crystal structure of Dicer is necessary to authenticate this model.

Guide RNAs and RNA-Induced Silencing Complex

Hammond et al. (96) determined that the endogenous genes of *Drosophila* S2 cells could be targeted in a sequence-specific manner by transfection with dsRNA, and loss-of-function phenotypes were created in cultured *Drosophila* cells. The inability of cellular extracts treated with a Ca^{2+}-dependent nuclease (micrococcal nuclease, which can degrade both DNA and RNA) to degrade the cognate mRNAs and the absence of this effect with DNase I treatment showed that RNA was an essential component of the nuclease activity. The sequence-specific nuclease activity observed in the cellular extracts responsible for ablating target mRNAs was termed the RNA-induced silencing complex (RISC) (96).

After partial purification of crude extracts through differential centrifugation and anion exchange chromatography, the nuclease cofractionated with a discrete ≈25-nucleotide RNA species. These results suggested that small RNAs were associated with sequence-specific nuclease and served as guides to target specific messages based upon sequence recognition. In another report, the multicomponent RNAi nuclease was purified to homogeneity as a ribonucleoprotein complex of ≈500 kDa (97). One of the protein components of this complex was identified as a member of the Argonaute family of proteins and was termed Argonaute2 (AGO2). AGO2 is homologous to RDE1, a protein required for dsRNA-mediated gene silencing in *C. elegans*. AGO2 is a ≈130-kDa protein containing polyglutamine residues, PAZ, and PIWI domains characteristic of members of the Argonaute gene family. The Argonaute family members have been linked both to the gene-silencing phenomenon and to the control of development in diverse species. The first link between Argonaute protein and RNAi was shown by isolation of *rde1* mutants of *C. elegans* in a screen for RNAi-deficient mutants. Argonaute family members have been shown to be involved in RNAi in *Neurospora crassa* (QDE3) as well as in *A. thaliana* (AGO1) (75).

Recently, two independent groups identified additional components of the RISC complex. Hammond and group showed the presence of two RNA binding proteins, the Vasa intronic gene and dFMR proteins, in the RISC complex isolated from *Drosophila* flies (35). Of these, dFMR is a homologue of the human fragile X mental retardation protein. In a parallel study, Siomi and group also isolated a novel ribonucleoprotein complex from the *Drosophila* lysate that contained dFMR1, AGO2, a *Drosophila* homologue of p68 RNA helicase (Dmp68), and two ribosomal proteins, L5 and L11, along with 5S rRNA (106). Both of these groups showed not only the presence of these components in the RISC complex, but also interactions among these proteins in vitro. Other components of RISC have not been clearly established yet. Nevertheless, some of the proteins mentioned below could very well constitute the RISC complex.

RNA and DNA Helicases

Aberrant RNA elimination surveillance seems to be common to most eukaryotic organisms. However, a diverse array of proteins specific for each organism seem to carry out such surveillance. Broadly, they fall in the biochemically similar group of RNA-DNA helicases. A mutant strain (*mut6*) of *C. reinhardtii* was isolated in which a gene required for silencing a transgene was disrupted (232). This RNAi-resistant mutant also showed an elevated transposition activity. The *mut6* gene was cloned and sequenced. The deduced MUT6 protein contains 1,431 amino acids and is a member of the DEAH box RNA helicase family. It also has a glycine-rich region that includes several RGG repeats, resembling an RGG box, a motif implicated in RNA binding and protein-protein interactions. MUT6 also has three putative nuclear localization signals and is predicted to be nuclear by PSORT analysis (161). MUT6 RNA helicase may be involved in degradation of misprocessed aberrant RNAs and thus could be a part of an RNAi-related surveillance system.

In *Neurospora crassa*, three classes of quelling-defective mutants (*qde1*, *qde2*, and *qde3*) have been isolated (46). The *qde3* gene has been cloned, and the sequence encodes a 1,955-amino-acid protein (48). The protein shows homology with several polypeptides belonging to the family of RecQ DNA helicases, which includes the human proteins for Bloom's syndrome and Werner's syndrome (238). In addition, QDE3 is believed to be involved in the activation step of gene silencing. The DNA helicase activity of QDE3 may function in the DNA-DNA interaction between introduced transgenes or with a putative endogenous gene required for gene-silencing activation by unwinding the double-stranded DNA. These interactions may induce changes in methylation or chromatin structure, producing an altered state that could result in aberrant RNA production. Thus, QDE3 protein may be more important for the transcriptional part of gene silencing, i.e., TGS.

When the RNAi sensitivity of several existing *C. elegans* mutants was examined, two mutant strains, *mut2* and *mut7*, that had previously shown elevated levels of transposon mobilization also showed resistance to RNAi. Ketting et al. (116) identified a mutator gene, *mut7*, in *C. elegans* and character-

ized it at the molecular level. MUT7 was found to be homologous to proteins with 3′-5′ exonuclease domains, such as Werner's syndrome protein and *E. coli* RNase D. It contained all the key catalytic residues for nuclease activity. A model was proposed in which MUT7 was speculated to play a role in repressing transposition by degrading the target mRNA with its exonuclease activity.

smg (suppressor of morphological effects on genitalia) mutants of *C. elegans*, defective in a process called nonsense-mediated decay, have been isolated (63). Seven *smg* genes which are involved in nonsense-mediated decay have been identified (29, 100). Since this process also involves RNA degradation, the function of these genes, if any, in the RNAi process was examined. Animals mutant for a subset of these genes, *smg2*, *smg5*, and *smg6*, were initially silenced by dsRNA but later showed rapid recovery from the effects of RNAi, unlike the wild-type worms, which remained silenced. Thus, these genes might affect the persistence of RNA interference. On the other hand, *smg1*, *smg3*, and *smg4* mutant animals behaved like wild-type worms and did not recover from RNAi at all, indicating that these genes are not required for RNAi persistence. The *smg5* and *smg6* genes have not been cloned, but the *smg2* gene shows homology to *Saccharomyces cerevisiae upf1*, which encodes an ATPase with RNA-binding and helicase activities.

The SMG proteins could unwind dsRNA to provide a template for amplification activity. In this way, the three SMG proteins might facilitate amplification of the silencing signal and cause persistence of the silenced state. Alternatively, SMG proteins could increase the number of dsRNA molecules by promoting endonucleolytic cleavage of existing dsRNA molecules, which has been observed in *Drosophila* flies. No SMG2 homologues have been identified in plants or fungi. However, a search of the *A. thaliana* genome sequence database revealed a number of candidates with either helicase and/or RNase domains.

In a recent report, Tijsterman et al. (208) showed that unlike sense oligomers, single-stranded oligomers of antisense polarity could induce gene silencing in *C. elegans*. The antisense RNA-induced gene silencing was explained by proposing that RNA synthesis was primed on the mRNA by antisense RNA, resulting in dsRNAs, which acted as substrates for Dicer-dependent degradation. Antisense RNAs showed a requirement for the mutator/RNAi genes *mut7* and *mut14* but acted independently of the RNAi genes *rde1* and *rde4* of *C. elegans*. The *mut14* gene was cloned by genetic mapping and subsequent candidate gene approach. The MUT14 protein is a member of the family of putative RNA helicases that contain the signature DEAD box motif. These proteins are involved in diverse cellular functions. The helicase activity of MUT14 might thus act to permit de novo RNA synthesis on the target.

Dalmay et al. (54) identified an *sde3* locus in *A. thaliana* plants which is required for the PTGS phenotype. They proposed that SDE3 protein might be involved in the production of dsRNA. SDE3 differs markedly from QDE3/MUT7 and has slight similarity to MUT6 in the helicase motif. Although it is highly similar to Upf1p and SMG2, it is unlikely that SDE3 is the functional homologue of Upf1p and SMG2 because it lacks important motifs (167). Notably, no SDE3 homologue was found in *C. elegans*, suggesting that SDE3-like proteins are regulators rather than essential cofactors of PTGS and are not used in *C. elegans*. This is further supported by the observation that *sde3* mutant plants exhibit only partial loss of PTGS (55). The closest homologue of SDE3 as identified by BlastP was a mouse protein encoded by *gb110* (91, 159). These SDE3 homologues have RNA helicase motifs that are quite distinct from those of the DEAD, DEAH, and Ski2p types of RNA helicase (134). It has been speculated that SDE3 and SMG2 are multifunctional RNA helicases involved in PTGS.

Translation Initiation Factor

Mutants of *C. elegans* showing resistance to dsRNA-mediated RNAi were selected by Tabara et al. (203). They genetically mapped seven mutant strains that were placed in four complementation groups. One of the groups, *rde1*, consisted of three alleles. Gene *rde1* is a member of a large family which includes *Drosophila* homologues (*piwi* and *sting*) and *Arabidopsis* homologues (*argonaute* and *zwille*) and rabbit *eIF2C*. The full-length cDNA sequence for *rde1* was determined, and the deduced protein, consisting of 1,020 amino acids, was referred to as RDE1. The RDE1 protein is homologous to the product of the quelling deficiency (*qde2*) gene in *Neurospora crassa* (75). The initiation step of RNAi might be affected in the *rde1* mutant, as it completely lacks an interference response to several dsRNAs. It does not show any increase in transposon mobilization and or any effect on growth and development.

RNA-Dependent RNA Polymerase

The effects of both RNAi and PTGS are potent and systemic in nature. This has led to a proposed mechanism in which RNA-dependent RNA polymerases (RdRPs) play a role in both triggering and amplifying the silencing effect. Transgenic and virus-infected plants show an accumulation of aberrant transgenic and viral RNAs. The RdRP enzymes might recognize these aberrant RNAs as templates and synthesize antisense RNAs to form dsRNAs that are finally the targets for sequence-specific RNA degradation (45, 47, 56, 133).

Genetic screens of *Neurospora crassa* (QDE1) (48) and *A. thaliana* (SDE1/SGS2) (54, 160) led to the identification of proteins which are similar to tomato RdRP (77, 187) and are required for quelling and PTGS, respectively. This testifies to the importance of RdRP in gene silencing. Cogoni et al. (45) cloned the *qde1* gene from *N. crassa*. It encodes a 158-kDa protein which lacks the typical signal peptide or a transmembrane domain, indicating its intracellular location. Dalmay et al. (54) found that the 113-kDa *Arabidopsis* RdRP is encoded by *sde1*. It is a plant homologue of QDE1 in *N. crassa* and EGO1 in *C. elegans*, which are required for quelling and RNAi, respectively. The SDE1 protein is required for transgene silencing but not for virus-induced PTGS, suggesting that SDE1 might be required to produce dsRNA, the initiator of PTGS (54).

The dsRNA produced as an intermediate in virus replication by virus-encoded RdRP might induce PTGS itself, and thus SDE1 may not be required for virus-induced PTGS. Plants with the *sde* mutation grow and develop normally, excluding a role for *sde* in development or basic cellular function. Two PTGS-controlling genes, *sgs2* and *sgs3*, were identified in *A.*

TABLE 2. Components of posttranscriptional gene silencing

Phenomenon	Organism	Mutation causing defective silencing	Gene function	Developmental defect
Posttranscriptional gene silencing	Plant (*Arabidopsis thaliana*)	*sgs2*/*sde1*	RdRP	None
		sgs3	Unknown function	None
		sde3	RecQ helicase	
		ago1	Translation initiation factor	Pleiotropic effects on development & fertility
		caf1	RNA helicase & RNase III	
Quelling	Fungus (*Neurospora crassa*)	*qde-1*	RdRP	None
		qde-2	Translation initiation factor	None
		qde-3	RecQ DNA helicase	
RNA interference	Worm (*Caenorhabditis elegans*)	*ego-1*	RdRP	Gametogenetic defect & sterility
		rde-1	Translation initiation factor	None
		rde-2, *rde-3*, *rde-4*, *mut-2*	Unknown function	None
		K12H4.8 (*dcr-1*)	Dicer homologue RNA helicase & RNase III	Sterility
		mut-7	Helicase & RNase D	None
		mut-14	DEAD box RNA helicase	
		smg-2	Upflp helicase	
		smg-5	Unknown function	
		smg-6	Unknown function	
		sid-1	Transmembrane protein	
	Alga (*Chlamydomonas reinhardtii*)	*mut-6*	DEAH box RNA helicase	

thaliana by another group of workers (160). Later, it was found that *sgs2* and *sde1* are different descriptions of the same gene. On comparing the protein sequence of all the RdRPs, a conserved block was identified which seems to be crucial for RdRP function in PTGS and RNAi. *sgs3* mutants have the same molecular and phenotypic characteristics as *sgs2* mutants, but the SGS3 protein shows no significant similarity with any known putative proteins.

In *C. elegans*, EGO1, a protein required for RNAi, was found to be similar to tomato RdRP and the QDE1 protein of *Neurospora crassa* (197), as mentioned earlier. For a number of germ line-expressed genes, *ego1* mutants were resistant to RNA interference. The *ego1* transcript is found predominantly in the germ line. *ego1* is thus yet another example of a gene encoding an RdRP-related protein with an essential developmental function. RdRP is speculated to play a role in the amplification of the dsRNA signal, allowing its spread throughout the organism (50, 77, 168, 221). The RdRP is also perhaps responsible for sustaining PTGS at the maintenance level even in the absence of the dsRNA that initiates the RNAi effect.

In spite of its omnipresence in different kinds of eukaryotic cells, RdRP homologues are not coded by either the *Drosophila* or human genome. Though the systemic characteristics of RNAi have not been revealed yet in either flies or humans, the amplification of siRNAs may be an essential step of RNAi even in these systems. Hence, it is important to know how these steps of RNAi are biochemically carried out in the absence of RdRP activity.

Transmembrane Protein (Channel or Receptor)

The systemic spread of gene silencing from one tissue to another has been well established in *C. elegans* and plants. To investigate the mechanism of systemic RNAi, Winston et al. (231) constructed and used a special transgenic strain of *C. elegans* (HC57). They identified a systemic RNA interference-deficient (*sid*) locus required to transmit the effects of gene silencing between cells with green fluorescent protein (GFP) as a marker protein. Of the 106 *sid* mutants belonging to three complementation groups (*sid1*, *sid2*, and *sid3*), they isolated and characterized *sid1* mutants. The *sid1* mutants had no readily detectable mutant phenotype other than failure to show systemic RNAi. Interestingly, these mutants also failed to transmit the effect of RNAi to the progeny.

The SID1 polypeptide is predicted to be a 776-amino-acid membrane protein consisting of a signal peptide and 11 putative transmembrane domains. Based on the structure of SID1, it was suggested that it might act as a channel for the import or export of a systemic RNAi signal or might be necessary for endocytosis of the systemic RNAi signal, perhaps functioning as a receptor. No homologue of *sid1* was detected in *D. melanogaster*, which may be consistent with the apparent lack of systemic RNAi in the organism (80, 174). However, the presence of SID homologues in humans and mice might hint at the systemic characteristics of RNAi in mammals.

Genetic Mutations with Unknown Function

The three other complementation groups identified by Tabara et al. (203) in *C. elegans* are *rde2* and *rde3*, with one allele each, and *rde4*, with two alleles. *rde4* mutants behaved like the *rde1* strain in not showing any increase in transposon mobilization and no effect on growth and development. The product of *rde2* remains to be identified. *mut2*, *rde2*, and *rde3* exhibited high-level transposition similar to *mut7*. This suggests a possible biological role of RNAi in transposon silencing (203).

Mello and colleagues (87) have proposed that *rde1* and *rde4* respond to dsRNA by producing a secondary extragenic agent that is used by the downstream genes *rde2* and *mut7* to target

specific mRNAs for PTGS. According to this view, *rde1* and *rde4* act as initiators of RNAi whereas *rde2* and *mut7* are effectors. Various components of gene silencing have been listed in Table 2.

MECHANISM OF RNA INTERFERENCE

As the various pieces of the RNAi machinery are being discovered, the mechanism of RNAi is emerging more clearly. In the last few years, important insights have been gained in elucidating the mechanism of RNAi. A combination of results obtained from several in vivo and in vitro experiments have gelled into a two-step mechanistic model for RNAi/PTGS. The first step, referred to as the RNAi initiating step, involves binding of the RNA nucleases to a large dsRNA and its cleavage into discrete ≈21- to ≈25-nucleotide RNA fragments (siRNA). In the second step, these siRNAs join a multinuclease complex, RISC, which degrades the homologous single-stranded mRNAs. At present, little is known about the RNAi intermediates, RNA-protein complexes, and mechanisms of formation of different complexes during RNAi. In addition to several missing links in the process of RNAi, the molecular basis of its systemic spread is also largely unknown.

Processing of dsRNA into siRNAs

Studies of PTGS in plants provided the first evidence that small RNA molecules are important intermediates of the RNAi process. Hamilton and Baulcombe (92), while studying transgene-induced PTGS in five tomato lines transformed with a tomato 1-aminocyclopropane-1-carboxyl oxidase (ACO), found accumulation of *aco* small RNAs of 25 nucleotides. More direct evidence about the generation of siRNAs in RNAi came from an in vitro cell-free system obtained from a *Drosophila* syncytial blastoderm embryo by Tuschl et al. (212). These authors were able to reproduce many of the features of RNAi in this system. When dsRNAs radiolabeled within either the sense or the antisense strand were incubated with *Drosophila* lysate in a standard RNAi reaction, 21- to 23-nucleotide RNAs were generated with high efficiency. Single-stranded ^{32}P-labeled RNA of either the sense or antisense strand was not efficiently converted to 21- to 23-nucleotide products. The formation of the 21- to 23-nucleotide RNAs did not require the presence of corresponding mRNAs.

The role of the small RNAs in RNAi was confirmed independently by Elbashir et al. (69), who showed that synthetic 21- to 23-nucleotide RNAs, when added to cell-free systems, were able to guide efficient degradation of homologous mRNAs. To assess directly if the siRNAs were the true intermediates in an RNAi reaction, Zamore et al. (240) fractionated both the unprocessed dsRNAs and processed dsRNAs from the *Renilla luc* dsRNA-treated cell-free *Drosophila* system and showed that only the fractions containing native siRNAs were able to bring about the cognate RNA degradation and their ability to degrade RNA was lost when these fractions were treated at 95°C for 5 min. These in vivo and in vitro studies thus provided the evidence that siRNAs are the true intermediates of the RNAi reaction.

Together with the experiments to identify siRNAs as the key molecules for the RNAi effect, several investigators carried out the logical search for polypeptides that could generate such molecules. Based on the binding and cleavage properties of *E. coli* RNase III enzymes, Bass (13) for the first time predicted the involvement RNase III-type endonucleases in the degradation of dsRNA to siRNAs. The RNase III enzyme makes staggered cuts in both strands of dsRNA, leaving a 3′ overhang of 2 nucleotides. The first evidence for the involvement of RNase III enzyme in RNAi was provided by T. Tuschl's group, who chemically analyzed the sequences of the 21- to 23-nucleotide RNAs generated by the processing of dsRNA in the *Drosophila* cell-free system. They showed the presence of 5′-phosphate, 3′-hydroxyl, and a 3′ 2-nucleotide overhang and no modification of the sugar-phosphate backbone in the processed 21- to 23-nucleotide RNAs (69).

Two groups recently identified candidate enzymes involved in degradation by scanning the genomes of *D. melanogaster* and *C. elegans* for genes encoding proteins with RNase III signatures (17, 115). Bernstein et al. (17) showed that one of these identified genes, *dicer* in *Drosophila*, codes for the RNA processing enzyme that fragments dsRNA into 22-nucleotide fragments in vitro. An antiserum raised against Dicer could also immunoprecipitate a protein from the *Drosophila* extract or from S2 cell lysate, and these Dicer protein immunoprecipitates were able to produce RNAs of about 22 nucleotides from the dsRNA substrate. The direct correspondence in size of these RNAs with those generated from dsRNA by cell extract suggested a role of this protein in dsRNA degradation. The role of Dicer in RNAi was further confirmed by the fact that the introduction of Dicer dsRNA into *Drosophila* cells diminished the ability of the transfected cells to carry out RNAi in vitro. Similar experimental studies were carried out with *C. elegans* extract, and an ortholog of Dicer named DCR1 was identified.

A number of in vivo and in vitro experimental studies have shown that the production of 21- to 23-nucleotide RNAs from dsRNA requires ATP. The rate of 21- to 23-nucleotide RNA formation from corresponding dsRNAs has been shown to be six times slower in the *Drosophila* extract depleted for ATP by treatment with hexokinase and glucose (165). Bernstein et al. (17) and Ketting et al. (115) showed that the Dicer immunoprecipitates from *D. melanogaster* as well as S2 cell extracts and DCR1 immunoprecipitates from *C. elegans* extract required ATP for the production of 22-nucleotide RNAs (17, 115). Recently, Nykanen et al. (165) reduced ATP levels in *Drosophila* extract by 5,000-fold with a sensitive ATP depletion strategy and showed considerable reduction in the rate of siRNA production in the *Drosophila* cell extract. These experiments suggest that ATP controls the rate of siRNA formation. However, it is still unclear whether ATP is absolutely rate limiting for the production of siRNAs from dsRNA.

The RNase activity and dsRNA binding of 218-kDa recombinant human Dicer have also been examined in vitro (175). The enzyme generated siRNA products from dsRNA quite efficiently in the presence of Mg^{2+} and the absence of ATP. The RNase activity was sensitive to ionic interactions, whereas the dsRNA binding was quite effective in presence of high salt and did not require Mg^{2+} at all. The dsRNA binding domain is located at the C terminus of Dicer, which is separable from the helicase and PAZ motifs. Human Dicer expressed in mammalian cells colocalized with calreticulin, a resident protein of

the endoplasmic reticulum. In other systems, Dicer has also been found to complex with various other proteins (35, 106). Hence, it is possible that the Dicer RNase activity functions as a complex of proteins in vivo.

Amplification of siRNAs

One of the many intriguing features of RNA interference is the apparently catalytic nature of the phenomenon. A few molecules of dsRNA are sufficient to degrade a continuously transcribed target mRNA for a long period of time. Although the conversion of long dsRNA into many small siRNAs results in some degree of amplification, it is not sufficient to bring about such continuous mRNA degradation. Since mutations in genes encoding RNA-dependent RNA polymerase (RdRP) affect RNAi, it was proposed that this type of polymerase might replicate siRNAs as epigenetic agents, permitting their spread throughout plants and between generations in *C. elegans*. Recent studies by Lipardi et al. (135) and Sijen et al. (193) provided convincing biochemical and genetic evidence that RdRP indeed plays a critical role in amplifying RNAi effects.

Lipardi et al. (135), while investigating the dsRNA-dependent degradation of target mRNA in a *Drosophila* embryo cell extract system, showed the generation of full-length cognate dsRNAs from labeled siRNAs at early time points. Both single-stranded RNAs (equivalent to target mRNA) and dsRNAs served as templates for copying by RdRP. New full-length dsRNAs were formed rapidly and cleaved. They also showed a strict requirement for the 3′-hydroxyl group and 5′-phosphate group on siRNAs for primer extension in the RdRP-mediated reaction (135).

Sijen et al. (193) further revealed the role of RdRP activity in RNAi. In an RNAi reaction, they observed the formation of new siRNA species corresponding to target mRNAs but different from trigger dsRNAs. They named these new siRNAs secondary siRNAs. With a primary trigger dsRNA specific for the *lacZ* region of the target mRNA that encoded a GFP-LacZ fusion protein, these authors demonstrated the degradation of a separate GFP mRNA target. This kind of RNAi induced by secondary siRNAs was named transitive RNAi. These authors demonstrated the requirement for the *rrf1* gene, a *C. elegans* gene with sequence homology to RdRP, in the generation of secondary siRNAs and transitive RNAi (193).

Amplification of siRNAs might occur at various stages of the RNAi reaction and has been documented in plants, *C. elegans*, *N. crassa*, and *Dictyostelium discoideun* but not in flies and mammals (66). Though the RdRP activity is present in *Drosophila* embryo extract, as mentioned earlier, it is surprising that the fly genome does not code for RdRP. Additionally, numerous experiments also suggest that RdRP is not required for RNAi in *D. melanogaster* (98).

Degradation of mRNA

In the effector step of RNAi, the double-stranded siRNAs produced in the first step are believed to bind an RNAi-specific protein complex to form a RISC. This complex might undergo activation in the presence of ATP so that the antisense component of the unwound siRNA becomes exposed and allows the RISC to perform the downstream RNAi reaction. Zamore and colleagues (240) demonstrated that a ≈250-kDa precursor RISC, found in *Drosophila* embryo extract, was converted into a ≈100-kDa complex upon being activated by ATP. This activated complex cleaved the substrate. The size and constitution of the precursor as well as the activated RISC might vary depending on the choice of system (98). The antisense siRNAs in the activated RISC pair with cognate mRNAs, and the complex cuts this mRNA approximately in the middle of the duplex region.

A few independent studies demonstrated the importance of the RISC complex in this part of RNAi reactions. The mRNA-cleaving RNA-protein complexes have also been referred to as siRNP (small interfering ribonucleoprotein particles). It is widely believed that this nuclease is probably different from Dicer, judging from the substrate requirements and the nature of the end products. Since the target cleavage site has been mapped to 11 or 12 nucleotides downstream of 5′ end of the guide siRNA, a conformational rearrangement or a change in the composition of an siRNP ahead of the cleavage of target mRNA is postulated. Finally, the cleaved mRNAs are perhaps degraded by exoribonucleases (96).

A part of cleaved fragments of mRNA at the end of step 2 might also be converted to the duplex forms by the RdRP-like activity. These forms might have siRNA-like functions and eventually enter the pool of the amplification reaction. Thus, it is likely that amplification of the RNAi reaction takes place at both step 1 and step 2 of RNAi. In another model, it has been proposed that siRNAs do not act as primers for the RdRP-like enzymes, but instead assemble along the length of the target RNA and are then ligated together by an RNA ligase to generate cRNA. The cRNA and target RNA hybrid would then be diced by the DCR protein. All these models were summarized by Schwarz et al. (189). Most of the steps involved in the mechanism of RNAi have been illustrated schematically in Fig. 2.

RNA SILENCING FOR GENOME INTEGRITY AND DEFENSE

Considerable evidence indicates that PTGS has evolved as a protective mechanism against parasitic DNA sequences such as transposons and the RNA sequences of plant viruses. DNA methylation and transcriptional gene silencing (TGS) are mainly responsible for keeping the transposition frequency at a minimum. However, PTGS also provides additional protection against the genomic instability caused by transposons. Mutations in the *C. elegans mut-7* gene increase the transposition frequency in the germ line and downregulate RNAi as well (58), implicating RNAi in the control of transposons. Recently, Djikeng et al. (61) cloned and sequenced the siRNA products of an RNA interference event occurring in *Trypanosoma brucei*. By sequencing over 1,300 siRNA-like fragments, they observed abundant 24- to 26-nucleotide fragments homologous to the ubiquitous retrotransposon INGI and the site-specific retroposon SLACS. Thus, they convincingly demonstrated that RNAi is involved in silencing the retroposon transcript.

In plants, PTGS has been widely linked with RNA virus resistance mechanisms (219, 227). Plant RNA viruses are, in fact, both inducers and targets for PTGS and gene-silencing-

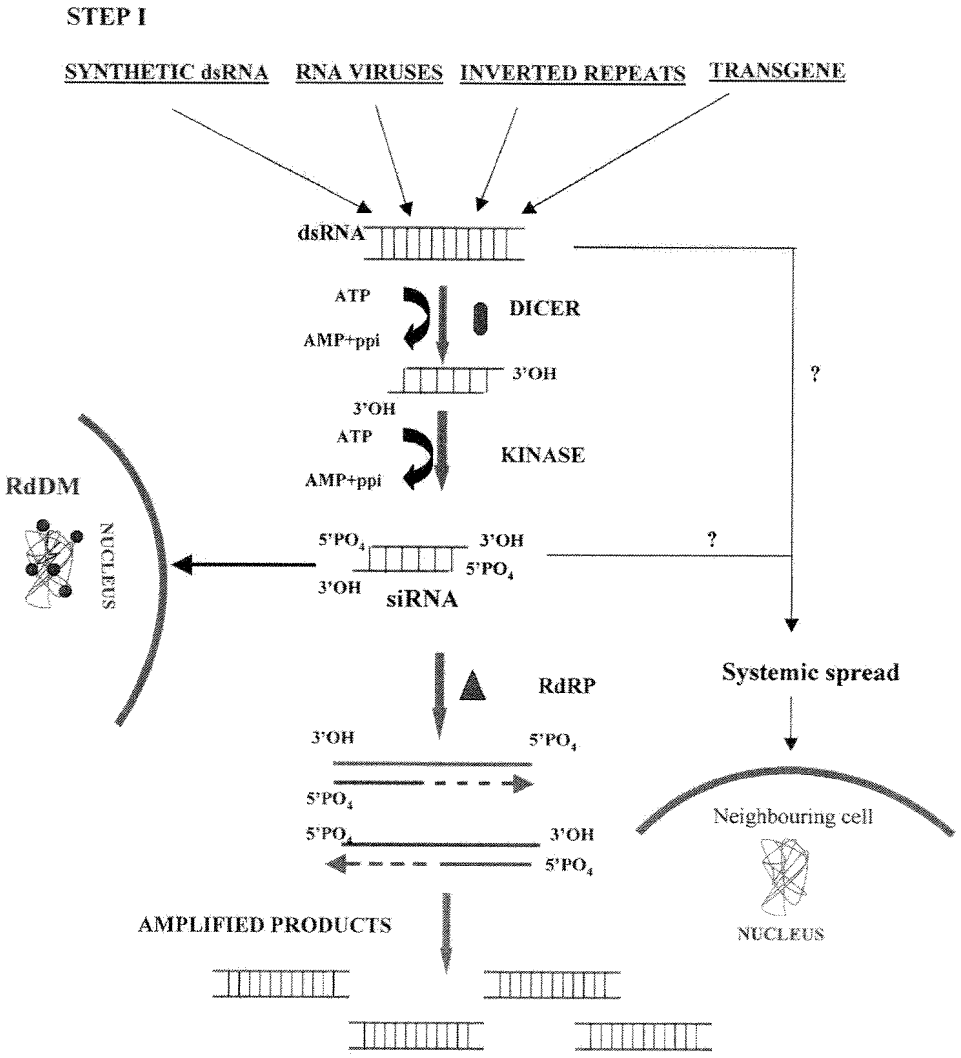

FIG. 2. Two-step model for the mechanism of gene silencing induced by double-stranded RNA. In step I, dsRNA is cleaved by the Dicer enzyme to produce siRNAs. A putative kinase seems to maintain 5′ phosphorylation at this step. The siRNAs have also been proposed to be responsible for nuclear DNA methylation (●) and systemic spread of silencing. Amplification might occur due to the presence of RdRP (▲). In step II, the siRNAs generated in step I bind to the nuclease complex (RISC). A helicase present in the complex might activate RISC by unwinding the siRNAs. The antisense component of siRNA in the RISC guides the complex towards the cognate mRNA (—), resulting in endonucleolytic cleavage (↓) of the mRNA. RdDM, RNA-dependent DNA methylation.

defective mutants of plants show increased sensitivity to viral infections (160). The direct role of dsRNA in inhibiting viral infection has recently been demonstrated by Tenllado and Diaz-Ruiz (207). They showed that dsRNAs derived from viral replicase sequences could interfere with virus infection in a sequence-specific manner by directly delivering the dsRNAs to leaf cells either by mechanical coinoculation with the virus or via an *Agrobacterium*-mediated transient-expression approach. Successful interference with the infection of plants by representative viruses belonging to the tobamovirus, potyvirus, and alfamovirus genera has been demonstrated. These results support the view that a dsRNA intermediate in virus replication acts as an efficient initiator of PTGS in natural virus infections.

The clinching support for the notion that PTGS has evolved as an antiviral mechanism has come from reports that plant viruses encode proteins that are suppressors of PTGS (8, 25, 222). These suppressors have evolved to save the viral RNA genomes from the PTGS degradative machinery of host plants. Different types of viral suppressors have been identified through the use of a variety of silencing suppression assays. Suppressors HC-PRO, P1, and AC2 are one type (encoded by potyviruses, rice yellow mottle sobemovirus, and geminiviruses of subgroup III, respectively) that is able to activate GFP expression in all tissues of previously silenced GFP-expressing plants (222). HC-PRO reduces target mRNA degradation and is thus responsible for reduced accumulation of siRNAs (137, 145). The second type of suppressors include movement proteins, i.e., p25 of potato virus X, which are involved in curbing the systemic aspect of transgene-induced RNA silencing (220). The third type includes cytomegalovirus 2b protein, which is

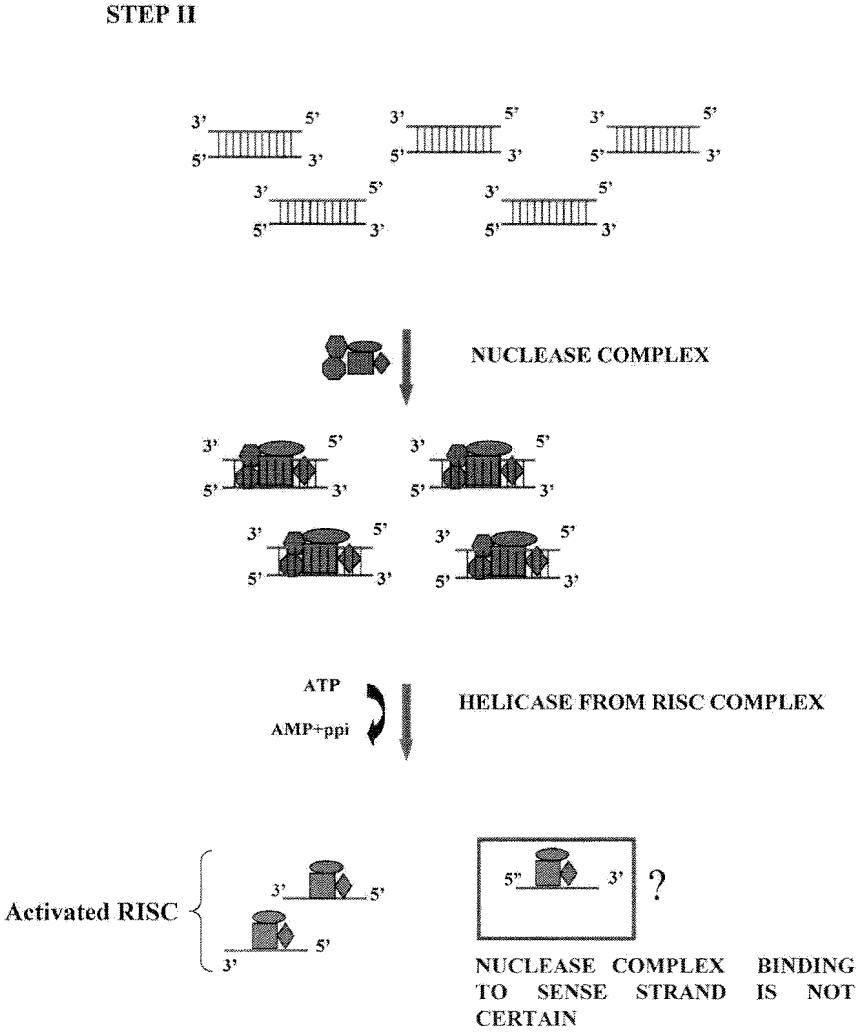

FIG. 2—*Continued.*

involved in systemic signal-mediated RNA silencing (60). The cytomegalovirus 2b protein is nucleus localized and also inhibits salicylic acid-mediated virus resistance (141). Other types of viral suppressors with undefined biochemical activities are also known (128). These findings not only provide the strongest support that PTGS functions as a natural, antiviral defense mechanism, but also offer valuable tools for dissecting the biochemical pathways of PTGS (128).

The PTGS degradative machinery can both detect and inactivate repetitive DNA sequences, suggesting it controls the expansion of repetitive elements, including endogenous genes (18). Although RNAi occurs in mammals and mammalian cell cultures, its role in animal virus protection is not clear. In mammals, dsRNA induces RNAi as well as interferon-mediated nonspecific RNA degradation and other nonspecific responses leading to blockage in protein synthesis and cell death (2). Thus, mammals seem to have evolved multiple mechanisms to detect and target dsRNA and to fight viruses. These various mechanisms may have different specificities or can function in distinct tissues or during development (210). A few other roles of RNAi in development and genome maintenance will be discussed in later sections.

MECHANISTIC DIFFERENCES AMONG THE BIOSYNTHETIC PATHWAYS OF siRNA

Although the functional parallelism of gene silencing is quite apparent in plants and animals, a few unique attributes separate the pathways in these groups. For example, systemic spreading of the RNAi reaction from the site of initiation is known to occur in plants and worms (74, 79), but not in flies or mammals. The noteworthy distinct molecules that have been identified to cause differences at the pre-Dicer, Dicer, and post-Dicer stages of gene silencing pathways are mentioned below.

Pre-Dicer stage. Plant proteins such as SGS2 (RdRP), SGS3 (coiled protein), AGO1 (responsible for plant development), and HEN1 (enhancer of floral *hua1* mutation) are required for

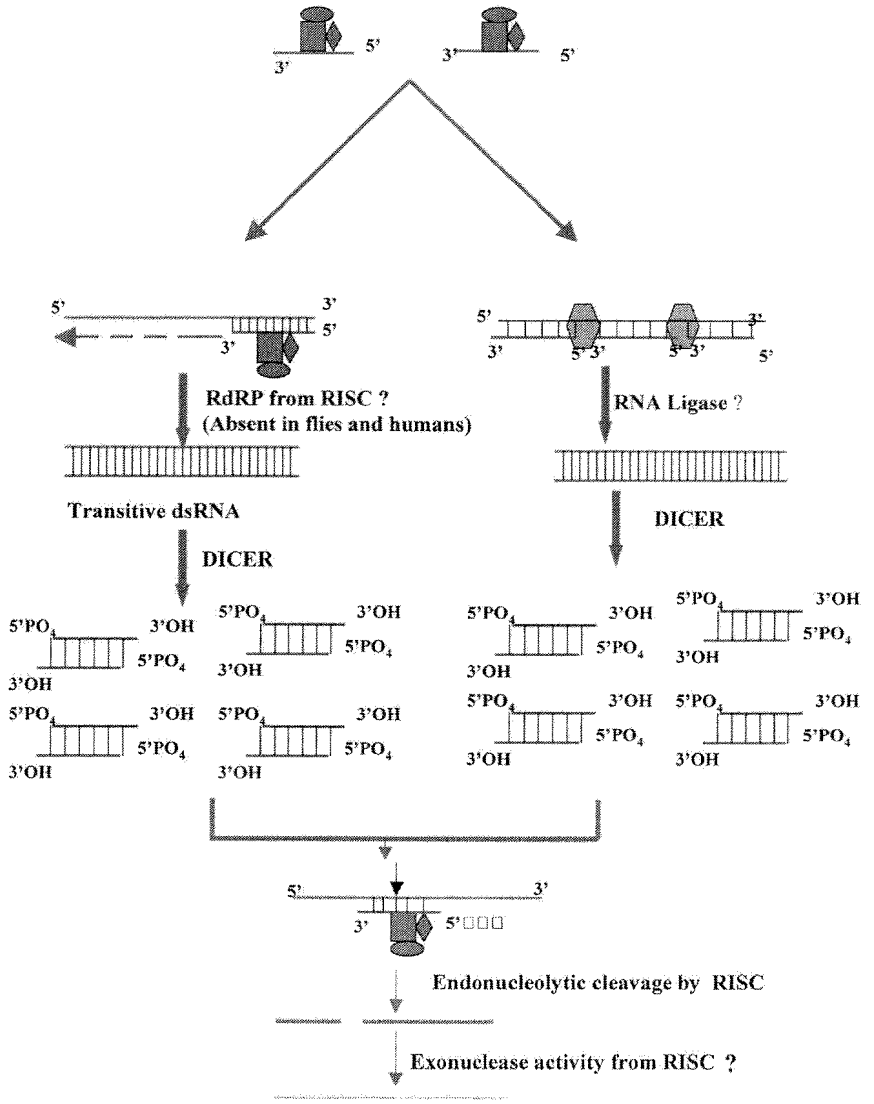

FIG. 2—*Continued.*

PTGS activities induced by the sense transgenes. But if the transgenes are in the form of hairpins expressing the panhandle dsRNA, the absence of or defects in the above-mentioned proteins do not play any role in altering the PTGS/cosuppression function. Hence, those proteins supposedly play a role upstream of dicing of dsRNA and may be involved in the formation and stabilization of dsRNA (22).

Homologues of SGS3 are unknown beyond the plant world. Even though HEN1 analogs are known in bacteria, yeasts, and animals, their roles in sense PTGS have not yet been identified. Likewise, SGS2 homologues are known in *C. elegans*, *N. crassa*, and *Dictyostelium discoideum*, but their roles at the pre-dicing stage have not been established yet in those systems. The equivalents of SGS2 in other animal systems are nonexistent both structurally and functionally (205). The role of worm AGO1 protein, i.e., RDE1, is also unique, as described earlier. AGO1 homologues are present in all eukaryotes, but they mostly function as a component (AGO2) of the animal RISC complex (32). The plant HEN1 protein is believed to be nuclear because of the presence of the nuclear localization signal at its N-terminal region (40). Since HEN1 is essential for plant PTGS (cosuppression), which is supposedly a cytoplasmic activity, the exploration of its subcellular distribution is of utmost importance. Boutet et al. (22) speculated that HEN1 could be a dsRNA stabilizing protein, and since many such proteins are known in the animal kingdom, it would be of interest to find animal analogs of the plant HEN1 protein. In fungus as well as the animal system, sense transgene-induced PTGS phenomena are known, but the machinery operative at the pre-dicing stage is still elusive.

The roles of plant SGS2, SGS3, AGO1, and HEN1 proteins may be limited at the stage of production of dsRNA from the transcript of sense transgenes, but no mechanism has been established regarding the presentation of the dsRNA to Dicer for the generation of siRNA. However, such a mechanism has been reported in *C. elegans*. The RDE4 and RDE1 (AGO1)

proteins of *C. elegans* were reported as initiators of RNAi and speculated to have no mechanistic role in the downstream processes of RNAi (87, 203). Unlike the *Arabidopsis* AGO1 and HEN1 proteins, RDE4 and RDE1 proteins are required for RNAi even when the dsRNAs are produced intracellularly in transgenic worms (203), but the defects in RDE4 and RDE1 are of no consequence if exogenous siRNAs or short antisense RNAs drive the RNAi reaction (208). RDE4 binds tightly to dsRNA (during the RNAi reaction) by virtue of its two RNA-binding domains and is always found in a tight complex with RDE1 protein even in absence of the RNAi reaction. During RNAi, RDE4 is found in a complex with RDE1, Dicer (DCR1), and a conserved DEXH-box RNA helicase (DRH1/DRH2). Based on these observations and other genetic evidence, Tabara and coworkers postulated that RDE4 and RDE1 functioned together to detect and retain foreign dsRNA and present the dsRNA to DCR1 for processing into siRNAs (202). Analogs of the RDE4 and DRH proteins are found in many eukaryotes, including plants and humans, but their roles have not been defined yet.

Dicer stage. The plant Dicer responsible for biosynthesis of plant siRNA is not known yet, whereas the Dicers of *C. elegans*, *D. melanogaster*, and humans as effectors for siRNA have been well characterized. The *A. thaliana* and rice genomes both encode at least seven RNase III-like proteins, of which at least four are putative homologues of Dicer, conveniently called DCLs (i.e., DCL1, DCL2, DCL3, and DCL4). The genetic evidence rules out that the *Arabidopsis* DCL1 (or CAF1) could be competent for siRNA formation (76). The roles of other DCL proteins are still to be revealed.

Interestingly, both in vivo and in vitro data suggest that the end products of plant dicing activities are different from those of the animal Dicers. When uniformly ^{32}P-labeled dsRNA was incubated with wheat germ extract, Zamore et al. (205) found that the dsRNA was chopped into siRNAs of two discrete size classes, one ≈21 nucleotides and the other 24 to 25 nucleotides long, whereas *D. melanogaster* and human Dicers generated only the 21-nucleotide siRNAs. Two similar size classes were also produced with cauliflower extract and were found independently in the set of 423 endogenous small RNAs cloned from *A. thaliana*. Thus, in plants, dicing activity leads to the generation of two distinct classes of siRNAs.

With specific synthetic siRNAs that supposedly bind tightly to and inhibit Dicer as competitors, Zamore et al. concluded that a different Dicer-like enzyme was responsible for the generation of each class of siRNA. These two distinct classes of siRNAs were reported first in vivo from transgenic plants bearing the silenced GFP sense transgenes (94). With an array of plant virus-encoded suppressors of gene silencing, Baulcombe et al. proposed that the 21-mer siRNAs controlled localized PTGS via mRNA degradation and the 24-mer siRNAs triggered systemic silencing and methylation of the homologous DNA. It remains to be seen whether this kind of dual dicing activity reflects any novel pathway intrinsic to plant RNAi. Interestingly, the longer (≈25-mer) siRNAs have also been detected in the natural RNAi biology of *Trypanosoma brucei* (61).

Post-Dicer stage. RISC has been isolated from *D. melanogaster*, *C. elegans*, and humans, and only some of its components have been characterized biochemically and genetically. Both mammalian and *Drosophila* RISC contain AGO2 proteins, whereas the GEMIN3 (a DEAD box helicase) and GEMIN4 proteins are found only in mammalian RISC (103). Similarly, dFXR, a homologue of the human fragile X mental retardation protein, is found only in *Drosophila* RISC (35). However, there is no report on the isolation of RISC in plants. Hence, mechanistically little is known about postdicing activity, especially in plants. A worthwhile question to address is whether there is any anchoring site for the occurrence of RNAi in the cytoplasm. Recently, it was reported that *E. coli* RNase III binds to the 70S ribosome and is functionally modified after binding (6). It is widely believed that the RISC associates with eukaryotic ribosomes (96). Hence, the exploration of ribosome association of the RNAi activities, especially of dicing and postdicing leading to mRNA degradation, might shed light on RNAi mechanisms in the future. The various affinities of ribosome-binding complexes might also reveal interesting system-specific features.

RdRP-dependent siRNA amplification and systemic spreading from the site of origin is another area where many system-specific variations have been noticed. RdRP homologues are not present in many organisms, so the mechanisms by which sense transgene-mediated PTGS are effected in those organisms remain a mystery (98). In other systems where RdRP is present, the biochemical steps and details of siRNA amplification may not necessarily be the same.

In *C. elegans*, RRF1 (a putative homologue of RdRP), along with other proteins, is required for RNAi even when the trigger dsRNA is expressed directly from the hairpin transgene in the nuclei of somatic tissues, whereas SGS2 (*Arabidopsis* RdRP) is dispensable for PTGS activity if induced directly by hairpin sense transgenes in *A. thaliana*. This suggests that the RdRP-mediated putative amplification steps of worms are different from those of plants (37). In plants, the SGS2-dependent spreading of silencing occurs from the region homologous to the trigger dsRNA into both the adjacent nonhomologous 5′ and 3′ regions of a target transgene (214). In contrast, spreading occurs only in the 5′ region in worms and fungi, which is consistent with the primer-dependent 5′-3′ copying activity of RdRP. Hence, in plants, the spread of silencing requires other activities (such as chromatin modification) in addition to that of RdRP (37).

In worms, tissue-specific variations of RdRP-dependent RNAi have also been reported, but not in plants or other systems. EGO1 is essential for RNAi in the germ line of *C. elegans*, whereas another RdRP homologue, RRF1, is required for silencing in soma (193, 197). Another intriguing observation is that the loss of function of RRF3 (third putative RdRP of worms) is responsible for the enhancement of sensitivity to RNAi in several tissues of *C. elegans*. Here, RRF3 acts as a negative regulator of RNAi, a fact difficult to reconcile with the postulated activity of RdRP (195). For systemic transmission of gene silencing, the membrane-bound SID1 protein of *C. elegans* and the plasmodesmatal connections of plants are implicated, but in both cases, the molecular nature of the moving signal has not been ascertained yet. An association between Dicer and the RdRP has been suspected in the case of *Dictyostelium discoideum* and *C. elegans*, but conclusive evidence is still lacking (37).

siRNA: SYNTHESIS, DELIVERY, AND GENE KNOCKDOWN

The natural RNAi biology of eukaryotic cells offers a protection mechanism against foreign nucleic acids; however, only in the recent past has the exploitation of its mechanistic details sparked a revolution in the investigation of cellular gene functions. Transcriptional regulation with the dsRNA technology provides an easy means to identify cellular characteristics in response to both internal and external cues. However, the application of RNAi in higher eukaryotes, particularly mammalian cells, has been hampered by the presence of a number of dsRNA-triggered pathways that mediate nonspecific suppression of gene expression (152). These nonspecific responses to dsRNA are not triggered by dsRNAs shorter than 30 bp, including the siRNA duplexes. Moreover, studies in *C. elegans* and *D. melanogaster* have clearly demonstrated that synthetic siRNAs can produce effects similar to those of the long dsRNAs (69, 236). Based on these experimental analyses, siRNAs are now being optimized for systematic exploration of the function of genes in a variety of organisms.

Prior to the siRNA era, approaches such as gene targeting by homologous recombination, ribozymes, and antisense technologies were commonly used to determine gene functions. All such approaches have their limitations, and none can be applied universally (201). The dawn of siRNA-directed knockdown approaches facilitated studies of gene function in a rapid and inexpensive way. This siRNA technology has the potential to decipher the function of virtually any gene that is expressed in a cell type- or pathway-specific manner. In the span of only a few years, large-scale functional analysis of almost all the ≈19,000 genes of *C. elegans* has been carried out with the siRNA-directed knockdown approach. A fairly detailed account of this technology has recently been reviewed by Dykxhoorn et al. (66).

Here, some of the salient aspects of the technology are summarized. In brief, the application of siRNA for gene silencing involves a careful consideration of the following variables: (i) selecting the siRNA sequence in the target gene; (ii) synthesis of siRNAs or construction of plasmids bearing DNA sequence encoding for siRNAs; (iii) optimizing transfection of the siRNAs or the plasmids expressing siRNAs in the target cells; and (iv) monitoring the efficacy of gene silencing.

Selection and Generation of siRNA

Several siRNAs synthesized against different regions of the same target mRNA show different silencing efficiencies (101). A number of groups have analyzed several parameters for optimizing siRNA-induced gene silencing, and these include the length, secondary structure, sugar backbone, and sequence specificity of the siRNA duplex. The efficacy of these parameters has been tested on several occasions for induction of RNAi in *D. melanogaster* and human cells (69, 189). No consensus on choosing the siRNA sequence has evolved. A line of thinking seems to suggest the following. The sequence should be selected in the region 50 to 100 bp downstream of the start codon. The 5′ or 3′ untranslated regions and regions near the start codon should be avoided, assuming that untranslated region-binding proteins and translation initiation complexes may interfere with the binding of siRNP or RISC endonuclease complex. The GC content of the siRNAs should be kept between 30 and 70%. The computer programs developed by Lin (Jack Lin's siRNA sequence finder; www.lc.sunysb.edu/stu/shiklin/rnai.html) and by Ambion (www.ambion.com) offer helpful guidelines to select potential siRNA sequences and determine whether these selected sequences match mRNA sequences other than those of intended target.

Based on different experimental approaches, a few guidelines have been laid for the synthesis of siRNAs. A general rule is that the sequence of one strand should be $AA(N_{19})TT$, where N is any nucleotide, i.e., these siRNAs should have a 2-nucleotide 3′ overhang of uridine residues. The siRNAs should be 21 nucleotides long. The siRNAs should have 5′-phosphate and 3′-hydroxyl group for efficiency. Compared to antisense or ribozyme technology, the secondary structure of the target mRNA does not appear to have a strong effect on silencing. The 21-nucleotide siRNAs can be chemically synthesized with appropriately protected ribonucleoside phosphoramidites and a conventional synthesizer and thus are widely available commercially. However, the use of chemically synthesized siRNA in RNAi has been restricted because of the high synthesis cost. Due to the paucity of information on the selection of siRNAs and their structures, these general guidelines are suggestive and do not guarantee the silencing effect. To overcome the siRNA selection ambiguity, Yang et al. (235) incubated dsRNA with the *E. coli* RNase III enzyme to generate a random array of siRNAs. The introduction of such a reaction soup resulted in the silencing of the target gene.

The exorbitant cost of synthesizing siRNAs and their lack of amplification in mammalian cells have compelled investigators to explore alternative strategies to generate a continuous supply of a battery of siRNAs. Several groups have devised strategies to synthesize short RNAs in vitro (64) or by introducing plasmids with the ability to make de novo siRNAs inside the cell (235, 239). DNA-based plasmid vectors have been designed by cloning siRNA templates downstream of an RNA polymerase III transcription unit, which normally encodes the small nuclear RNA U6 or human RNase H1.

Two approaches have been developed for expressing siRNAs. In the first, sense and antisense strands constituting the siRNA duplex are transcribed by individual promoters (64), and in the second, siRNAs are expressed as fold-back stem-loop structures that give rise to siRNAs with a small loop. A stretch of four to five thymidines is added at the end to the siRNA template that acts as a transcription termination signal. Many of these plasmid-based vectors, such as pSilencer 1.0 (Ambion) and pSuper (DNA Engine), are now commercially available. These vectors provide advantages over chemically synthesized siRNAs, but use of these plasmid vectors also remains limited due to numerous disadvantages, including the transient nature of siRNA expression and low as well as variable transfection efficiency.

To circumvent these problems, virus-based high-efficiency siRNA delivery systems are also being developed. A retrovirus-based system developed by Devree and Silver (59) is cited here as an example. The U6 promoter along with the siRNA-generating hairpin construct was cloned upstream of the 3′ long terminal repeat of the commercially available pMSCV-puro vector. The in vitro-packaged recombinant virus was allowed to

transfect HeLa cells with high efficiency in the presence of puromycin selection, and a dramatic downregulation of the target gene product was observed. A downregulation of this extent was not possible with the plasmid-based delivery system. Such virus-based vectors or their improved variants hold the promise to efficiently detect the function of any gene in virtually any cell type, provided that the production of recombinant virus is not a limitation.

Most of the siRNA expression vectors produced to date use RNA polymerase III regulatory units, which do not allow tissue-specific siRNA expression. However, Shiagawa and Ishiid reported a polymerase II promoter-based plasmid encoding a dsRNA expression system that could eventually express siRNA in a tissue-specific manner (192). In their novel scheme, a pDECAP vector was used, which expressed long dsRNAs corresponding to the *ski* gene (encoding a transcriptional repressor) in the form of a hairpin. The engineered hairpin RNA expressed from a cytomegalovirus promoter lacked the 7-methylguanosine cap structure at its 5′ end and a poly(A) tail at its 3′ end. The transcript of such a design did not exit the nucleus to reach the cytoplasm and thus prevent the interferon pathway-mediated nonspecific antiviral response. The double-stranded transcript was diced in the nucleus, and the siRNAs were subsequently released into the cytoplasm to mediate the gene-specific silencing. The silencing was specific, since the level of a related protein, SNO, remained unaffected.

The same vector was also used to create *ski* knockdown mice, the phenotype of which was similar to that of *ski* knockout embryos, which exhibited defects in neural tube and eye formation. Later generations of such vectors may use more tissue-specific *cis*-acting elements in the employed promoter to stringently knock down gene functions in the animal system. It is pertinent to highlight here that because plants do not elicit an interferon-mediated antiviral response, the dsRNA/siRNA delivery system need not be as complex as the pDECAP system.

Transfection of siRNA and Detection of Gene Silencing

An attempt to understand a gene's function in diverse organisms necessitates optimization of protocols for efficient delivery of siRNAs into cells. A number of transfection reagents are being employed for transfecting siRNA into different cell lines. Lipofectamine 2000 and Oligofectamine (Invitrogen) are being routinely used for siRNA delivery. A few newer transfection reagents such as TransIT-TKO (Mirus) and Ambion's Siport Amine and Siport, have also been used successfully in cultured cell lines. Electroporation has been used to transfect siRNAs in cell lines as well as in parasites such as *Trypanosoma brucei* and *Plasmodium falciparum* (150, 213). In adult mice, naked siRNAs have been delivered by hydrodynamic transfection methods to combat hepatitis C virus infection in the intact liver (151). The transfecting siRNAs have been used successfully for studying the role of proteins in DNA damage response and cell cycle control, general cell cycle metabolism, signaling, the cytoskeleton and its rearrangement during mitosis, membrane trafficking, transcription, and DNA methylation (211). These molecules have also been used to differentiate between housekeeping and other genes (112).

The preferred way to detect specific gene knockdown by RNAi is to study the depletion of the target protein by immunofluorescence and Western blotting with the specific antibody. In addition, the knockdown phenotype and Northern blot analysis can also be used to detect the effects of siRNA. If the gene is essential, cellular growth is delayed or arrested, and [^{3}H]thymidine uptake can also be used to assign the function of a particular gene (70).

siRNA Introduction into Plants

siRNAs have been delivered into tobacco plants by biolistic pressure to cause silencing of GFP expression. Silencing occasionally was detected as early as a day after bombardment, and it continued to potentiate up to 3 to 4 days postbombardment. Systemic spread of silencing occurred 2 weeks later to manifest in the vascular tissues of the nonbombarded leaves that were closest to the bombarded ones. After a month or so, the loss of GFP expression was seen in nonvascular tissues as well. RNA blot hybridization with systemic leaves indicated that the biolistically delivered siRNAs induced the de novo formation of siRNAs, which accumulated to cause systemic silencing (118).

MICRO-RNA

Since the RNAi machinery is present constitutively within eukaryotic cells, it is important to explore and understand the metabolic advantages that are accorded by RNAi-related proteins during the intrinsic normal growth of cells and development of organisms. The natural RNAi machinery not only keeps the mobile transposable elements from disrupting the integrity of genomes, as was suggested by analyses in lower plants, *A. thaliana*, *C. elegans*, *D. melanogaster*, and animals (9, 94, 138, 203, 232), but also participates in organism development. Genetic defects in *C. elegans* RNAi genes *ego1* and *dicer* cause known, specific developmental errors (87, 119, 197). Similarly, the Argonaute family of genes of *A. thaliana* (especially the ZWILLE proteins) is also responsible for plant architecture and meristem development (32), and the Dicer homologue of *A. thaliana*, CAF1, is required for embryo development (83). Thus, genetic evidence illustrates the role of the RNAi machinery as a controller of development-related genes. The mechanistic details of these developmental processes are beginning to emerge.

In 1991, Ambros and coworkers first isolated a *lin4* mutant of *C. elegans* which was arrested at the first larval stage (127). Later on, the *let7* mutation was isolated in the same system, which was responsible for development through the fourth larval stage. Both *lin4* and *let7* encode short 22-nucleotide mature RNAs and were called short temporal RNA because they control the temporal development program of *C. elegans*. The mature *lin4* RNA defines (negatively regulates) the mRNA expression of the *lin14* and *lin28* heterochronic genes with the antisense-mediated repression mechanism of translation initiation and thus specifies the fate of cells during the first three larval stages. Recent studies have revealed that the short temporal RNAs are actually members of a group of tiny RNAs (21 to 28 nucleotides) called the micro-RNAs, isolated members of which could easily run to a few hundreds. Some of the components of the RNAi machinery have also been clearly

established as the effector proteins for the maturation of micro-RNAs.

Identification and Biogenesis

A range of biochemical techniques have been applied to clone the 21- to 28-nucleotide RNAs that are present during the normal cellular development of many organisms, for exploring the abundance and complexity of micro-RNA. Micro-RNAs have been found to be abundant and phylogenetically extensive in plants, flies, worms, and humans. In *D. melanogaster*, *C. elegans*, plants, and humans, more than 600 micro-RNAs have been identified (123, 125, 126, 137). Bioinformatic analyses of the complete genome sequences have been extremely useful for identification studies. The genome sequences of a variety of organisms revealed the authenticity of these micro-RNAs, the nature of the precursor RNAs, the genomic locations of micro-RNA genes, and the evolutionarily conserved character of some of these micro-RNAs. With the RNA folding program mfold (148) and Northern analyses of micro-RNA, it has been universally inferred that most micro-RNAs arise from the imperfectly annealed 70-nucleotide hairpin precursor RNA whose expression is often developmentally regulated. These micro-RNAs are thus predicted to be processed from multiple bulged and partially duplex precursors, like the short temporal RNA precursors (186).

The identification of micro-RNAs is the first major hurdle in micro-RNA-related research. The first step in computational identification of micro-RNAs from genome sequences is identification of sequences forming hairpin loops (stem-loop sequences). For this purpose, software such as srnloop (85) and RNA fold (130) is used. These are Blast-like software packages which identify short complementary sequences within a specified distance on the genome. The hairpin sequences obtained by this analysis are then evaluated as candidate micro-RNAs based on different criteria, such as GC content and minimum free energy, and by passing through different filters, such as short-repeat filters and structure quality filters (85).

Another important criterion that has been used for the identification of candidate micro-RNAs is the correspondence of a hairpin of one species with that of another species. Two hairpins are said to be in correspondence if a short sequence (>19 nucleotides) in the stem of one hairpin is also present in the stem of another hairpin, although the two hairpins may have otherwise variable sequences. If a hairpin from one species has correspondence with a hairpin from one or more other species, this strengthens its status as a candidate to be a micro-RNA. The homology of hairpins with known micro-RNAs is also considered a useful criterion to select candidate micro-RNAs.

D. P. Bartel's laboratory has developed a computational procedure, called MiRscan, to identify micro-RNAs based on their homology to known micro-RNAs with respect to the characteristic features in the stem region. MiRscan evaluates the stem-loops by passing a 21-nucleotide window along the stem region and assigning a likelihood score to each window that measures how well its attributes resemble those of the previously experimentally identified and validated micro-RNAs (129, 130). The candidate micro-RNAs identified by these procedures are experimentally validated by Northern blot assay of total small RNAs with the stem region of the candidate as a probe or by a more sensitive PCR assay of the amplified small RNA library (85, 130). Detection of a 21- to 24-nucleotide band in these assays validates a candidate micro-RNA, whereas a ≈70-nucleotide band is detected in Dicer-deficient mutants, further confirming that the micro-RNAs arise from a ≈70-nucleotide precursor.

An analysis of micro-RNA expression in cell lines and tissues suggests cell- or tissue-specific expression. For example, micro-RNA 1 (miR1) is specifically expressed in human heart tissues and stage-specifically in mouse embryogenesis (126). *A. thaliana* small RNA 39 is detected exclusively in inflorescence tissues and downregulates the expression of a Scarecrow-like transcription factors (137, 139). Considering the diverse functions in which micro-RNAs have been implicated, micro-RNAs have also been named variously, i.e., micro-RNAs which mediate spatial development are referred to as sdRNAs, while cell cycle micro-RNAs are referred to as ccRNAs, etc. The regulated expression patterns of these micro-RNAs are suggestive of their functions in developmental control. However, many micro-RNAs are uniformly expressed, suggesting their role in general gene regulation (186). Downregulation of micro-RNAs leads to serious developmental defects, as evidenced by isolation of various micro-RNA mutants. Recent reports reveal that *miR15* and *miR16* are located in human chromosome 13q14, a region which gets deleted in more than half of B-cell chronic lymphocytic leukemias. Detailed deletion and expression analyses point out that these two micro-RNAs are located within a 30-kb region of loss in chronic lymphocytic leukemias, and both genes are deleted or downregulated in a majority (≈68%) of chronic lymphocytic leukemia cases (30).

A majority of micro-RNAs occur in relatively short (≈70-nucleotide) and single stem-loop precursor structures. However, in both animals and plants, some micro-RNAs are arranged in clusters. The genes in the tandem clusters are coexpressed, for example, in the germ line and early embryos of *C. elegans* and *D. melanogaster* (123, 125) and in the inflorescence tissues of *A. thaliana* (137). A set of seven highly related *C. elegans* micro-RNA genes that are coexpressed are so tightly clustered within 1-kb region that they are predicted to form a precursor from which all the seven mature micro-RNAs are processed (186). Similarly, several micro-RNAs originate from each of the five chromosomes of *A. thaliana* containing clusters of two to four micro-RNAs spaced irregularly within the intergenic region. Interestingly, three of the clusters contain micro-RNA sequences of both sense and antisense polarities, a scenario not found in the animal system yet. Such variations in the precursor structures of micro-RNAs may point towards distinct mechanisms of biosynthesis of micro-RNAs, although all micro-RNAs originate by transcription events that are independent of adjacent conventional genes.

Apoptosis-Related Micro-RNA

The proliferation of tissues and organs of any organism requires careful coordination between cell proliferation and cellular death. The proliferation processes of a cell include active inhibition of the apoptotic process. Recently, two micro-RNA genes, *bantam* and *mir14*, that suppress cell death by inhibiting the translation of apoptotic messages have been isolated from *D. melanogaster*. Expression of the *bantam* 21-nu-

cleotide micro-RNA is temporally and spatially regulated in response to patterning cues. The proapoptosis gene *hid* has been identified as a target for regulation by *bantam* micro-RNA (24). *bantam* deletion mutants grow poorly and die as early pupae, whereas *mir14* mutants are viable but stress sensitive and cursed with a reduced life span. The *mir14* suppresses death induced by expression of Rpr, Hid, Grim, or the apical caspase Dronc (234). *mir14* also regulates fat metabolism by decreasing the levels of triacylglycerol and diacylglycerol. *bantam* is related to *mir80* and *mir82* of *C. elegans*, indicating that the *mir80* family of RNAs might be involved in apoptosis in worms (33). Identification of these micro-RNAs promises discovery of similar micro-RNAs in other systems and reveals the hidden treasure of knowledge relating to micro-RNA-controlled biological functions (11).

Kinship of siRNA- and Micro-RNA-Related Pathways

Since micro-RNAs are derived from their precursor dsRNAs and are similar in size to siRNAs, the biogenesis of siRNAs and micro-RNAs is similar. In fact, both siRNAs and micro-RNAs are processed by Dicer activities in animals as well as in plants (86, 96, 104, 115, 240). Human recombinant Dicer can process pre-*let7* RNA to mature *let7* quite efficiently in vitro (175). Recent work by D. P. Bartel's group (181) has also shown that *caf1* (*dicer* homologue) mutants of *A. thaliana* fail to process micro-RNAs. The genetic and biochemical data point toward interaction between Dicer and the Argonaute group of proteins in *C. elegans* and *D. melanogaster* for processing the micro-RNAs (86, 95). The similar interaction is possibly also present in plants between Dicer on one hand and PNH (zwille/pinhead) on the other to generate plant micro-RNAs (83). Additionally, both forms of small RNA, micro-RNAs and siRNAs, were found integrally associated with riboprotein complexes containing a member of the PIWI/PAZ domain family, siRNAs in the RISC and micro-RNAs in the microribonucleoprotein complexes (96).

In fact, recent evidence suggests that at least for some micro-RNAs, the microribonucleoprotein and the RISC complex could be the same entity (103, 137, 139, 182). Though the same or similar DCR and subsequent ribonucleocomplexes are required to process mature forms of the micro-RNAs, in some cases, such as *C. elegans lin4* and *let7*, the ≈22-nucleotide form is processed from the 5′ part of the stem, and in other cases, such as *miR1* and *miR58*, maturation results from the 3′ part of the precursors. Thus, there is a gene specificity of micro-RNA processing and/or stabilization (126). Since the biosynthetic pathways of micro-RNAs and siRNAs are somewhat similar, the viral suppressors that inhibit siRNA formation are also expected to interfere in the biogenesis of micro-RNAs. A detailed understanding of this suppression process may unravel the hitherto unknown molecular basis of virus-induced development-related diseases in eukaryotes, especially in plants.

An RNA-silencing suppressor, PI/HC-PRO of turnip mosaic virus, induces a number of developmental defects in the vegetative and reproductive organs of *A. thaliana*. Many of these defects are reminiscent of observed defects in Dicer-like mutants of *A. thaliana*. The PI/HC-PRO suppressor interferes with the formation of *miR171*, and as a result the downstream target mRNAs accumulate instead of being cleaved, causing developmental errors (113). Thus, it is interesting that the counterdefensive strategy of the viruses has evolved not only to protect the viral RNA genome from the host degradative machinery but also to subvert the cellular development program in favor of the virus.

However, it is important to mark the distinctions among the pathways leading to the formation as well as the activities of siRNAs and micro-RNAs. Although over 600 micro-RNAs from various organisms have been identified (33), only about 3% of them are fully complementary to the target mRNA sequences. All known micro-RNAs are derived in vivo from dsRNA precursors which are imperfectly annealed. Since the biosynthesis and activities of the micro-RNAs do not require perfect complementarity, noncanonical pathways of RNAi may be involved for the micro-RNAs because the usual RNAi calls for extensive complementarity of the dsRNA. It is only because of this characteristic mismatch between the sequences of micro-RNA and cognate mRNA that the in silico identification of the target mRNA is so difficult (182). The imperfect nature of annealing between the two partners is viewed as the prime cause for translational repression of the target mRNA (172).

Second, the mature micro-RNAs are always found in the single-stranded conformation in nature for some unknown reason, whereas siRNAs are double-stranded when detected. Third, unlike siRNAs, micro-RNAs enter riboprotein complexes with differing PPD proteins (PAZ and Piwi domains), depending on the specificity of the micro-RNA or its precursor with the cognate PPD proteins (86). The sequence or structure of a micro-RNA or its precursor might ensure that it functions as a translational repressor and not as a trigger of RNAi. It is widely speculated that the siRNAs and micro-RNAs are distinguished following their biosyntheses, and these two are then allowed to form related but distinct ribonucleoprotein complexes that target downstream substrates for degradation or translation repression, respectively. This hypothesis is based on the observation that siRNAs or exogenously supplied hairpin RNAs containing even a single mismatch with their substrate fail to repress the target mRNAs and do not simply shift their regulatory mode to translation inhibition (98).

Fourth, a viral suppressor of RNA silencing, the HC-PRO protein of potato virus Y, has been found to differentially regulate the accumulation of siRNAs and micro-RNAs in tobacco (144). The HC-PRO protein prevents accumulation of siRNAs of the silenced genes and thus releases silencing in a universal manner, but the same protein helps accumulation of all micro-RNAs tested, namely, *miR167*, *miR164*, and *miR156* of tobacco, in vivo. This result indicates that the dicing complexes for siRNA and micro-RNA may not be exactly similar in biochemical features, and as a result the biochemical functions of the complexes are different in response to this particular HC-PRO protein. Lastly, not all RNAi pathway mutants are developmentally aberrant, whereas micro-RNA pathway mutants are expected to be defective in organism architecture and development. For example, an RNA-dependent RNA polymerase-defective mutant of *A. thaliana*, the *sgs2/sde1* mutant, shows defects only in cosuppression phenomena (a form of RNAi) but is perfectly normal in phenotypic development (54, 160). This observation raises the question of whether the

RdRP-dependent amplification step is required at all for micro-RNAs.

Functional Classifications

A number of micro-RNAs, including *let7*, are conserved across all organisms throughout evolution. About 12% of the micro-RNAs identified so far in animal systems are conserved at least among nematodes, *D. melanogaster*, and humans. Interestingly, a majority of the micro-RNAs are speculated to control development-related genes. However, the mechanisms of such control are not quite established yet. Micro-RNAs probably employ a variety of mechanisms to downregulate target genes. Micro-RNAs such as *C. elegans lin4* and *let7* have been shown to imperfectly anneal to the 3′ untranslated region of the target mRNA.

A vast majority of micro-RNAs probably belongs to this category. Due to imperfect complementarity, some micro-RNAs may also anneal to a host of different target mRNAs either simultaneously or in a temporally controlled manner. On the other hand, there are micro-RNAs, located mostly in the *A. thaliana* intergenic region, which have perfect or nearly perfect complementarity to the target mRNAs. Such micro-RNAs might trigger site-specific cleavage of the mRNA after being incorporated into a functional RISC-like complex. In such a situation, micro-RNAs act like siRNAs. The *A. thaliana* inflorescence-specific small RNA 39 cleaves the middle part of mRNA of the three scarecrow gene family members in a similar fashion (137).

On the basis of nearly perfect complementarity with the micro-RNAs, numerous *A. thaliana* mRNA targets have been predicted, and these targets have also been phylogenetically conserved in rice. Fifteen cleavage-type targets were validated recently by in vitro or in vivo micro-RNA-guided cleavage assays. The majority of these predicted mRNA targets encode members of large family of transcription factors, including Phavoluta (PHV), Phabulosa (PHB), cup-shaped cotyledon 1 (CUC1), CUC2, etc. These transcription factors are required for meristem identity, cell division, organ separation, and organ polarity (33). On the other hand, *mir172* likely acts in cell fate specifications as a translational repressor of APETALA2 in *Arabidopsis* flower development (39).

There are other varieties of micro-RNA which also interact with target mRNAs affecting the posttranscriptional steps, such as RNA splicing, mRNA localization, and RNA turnover. In *D. melanogaster*, many micro-RNAs are known to be complementary to the 3′ untranslated region sequence motifs, which are responsible for mediating negative posttranscriptional regulation. These sequence motifs include the K box (CUGUGAUA), the B-rd box (AGCUUUA), and the recently found GY box (UGUCUUCC). All micro-RNAs showing complementarity to these motifs are expressed either broadly throughout development or in the narrow window of embryogenesis of *D. melanogaster* (124). It is possible to have even a fourth class of micro-RNAs, which may serve as guides for modification of chromosomal DNA and control the epigenetic processes of nuclear genomes (225).

Genetic Diversity in Species-Specific Biosynthesis of Micro-RNA

The siRNA and micro-RNA pathways closely parallel each other. It has been mentioned earlier that the biosynthesis of siRNAs have interesting system-specific features. Hence, system-specific features of micro-RNAs would also be of no surprise. Here, we illustrate some of those features. In *C. elegans*, *D. melanogaster*, and other animals, the Dicer proteins responsible for siRNA formation are also involved in the biosynthesis of micro-RNAs; but in *A. thaliana*, DCL1 (or CAF1) is responsible for micro-RNA but not for siRNA formation (76). Interestingly, the DCL1 mRNA is predicted to be a micro-RNA target, indicating that the micro-RNA-related apparatus in plants is regulated by a negative feedback loop (233). In plants, HEN1 is required for both siRNA and micro-RNA formation (22). Such a role for HEN1 orthologues in other systems is not known yet. Both CAF1 and HEN1 have nuclear localization sequence signals, raising the question of whether plant micro-RNAs are made intranuclearly.

The functions of plant micro-RNAs may be different from those of their animal counterparts in some events. The animal micro-RNAs act as translational repressors, whereas some plant micro-RNAs act on the target mRNA posttranscriptionally, like siRNAs (139). In animals, the majority of the AGO family members tightly regulate the biosynthesis of micro-RNAs (32), whereas in plants, especially *A. thaliana*, only one member of the 10 constituents of the AGO family, Ziwelle, alone contributes to the synthesis of micro-RNA. Surprisingly, though *A. thaliana ago1* mutants show a strong hypermorphic phenotype, AGO1 protein is not responsible for plant micro-RNA formation (22). However, AGO1 is required for initiation of PTGS, whereas Zwielle is not. Recently, Vauchert et al. also isolated a few *ago1* alleles of *A. thaliana* which were hypomorphic in nature (157).

The biogenesis of some plant micro-RNAs seems to be different from that of their animal counterparts. Most of the *Arabidopsis* micro-RNAs belong to group1, as their precursor forms are detected poorly or not at all. Despite the absence or greatly reduced abundance of the mature micro-RNAs, accumulations of pre-micro-RNA are never detected in Dicer-defective *caf1* mutants (171). However, the pre-micro-RNAs accumulate to a higher level in *C. elegans* and metazoans in which Dicer activity is abolished or reduced (86). Very few *Arabidopsis* micro-RNAs belong to group II, including the micro-RNAs *miR176*, *-177*, *-178*, and *179*, the pre-micro-RNA transcripts of which are, however, detectable. The levels of these precursor transcripts do not change in either the *caf1* or *hen1* mutant background. Such facts indicate that even within the same plant, the biosynthesis pathways of micro-RNAs might vary depending on the particular micro-RNA. The tissue specificity of micro-RNAs is well known. Hence, micro-RNAs specific to tissues that are unique either to animals (e.g., brain) or plants (roots, for example) might exemplify variant pathways of biosynthesis of micro-RNAs.

The discovery of micro-RNAs has been branded one of the top discoveries in developmental molecular biology. The survey of micro-RNAs is still at a subsaturated stage. The future will witness the discovery of hundreds of new micro-RNAs and

their corresponding mRNA targets, and the mysteries of developmental pathways from embryogenesis to adulthood will be unfolded.

SMALL-RNA-MEDIATED EFFECTS ON CHROMOSOMAL DNA

The siRNAs work not only at the posttranscriptional stage but also leave their indelible marks on the genomes to repress the gene transcription activity or selectively remove portions of the genomes, especially of protozoans. These stunning discoveries have been reported only in the span of the last 2 years, the detailed mechanisms of which are still to be revealed and have been reviewed in two recent articles (57, 109). In the present review, we describe these effects briefly with special emphasis on plant systems, since the genetics and biochemistry of some of these processes are better illustrated in plants.

Broadly speaking, the siRNAs bring about three different biochemical end products with the chromatin DNA: DNA methylation, as revealed mostly in plant systems; heterochromatin formation; and programmed elimination of DNA. DNA methylation had been reckoned a major source of transcriptional gene silencing (TGS), and mechanistically TGS had been viewed very distinctively from PTGS in the past. But recent developments have caused a blurring in the identity between these two pathways (218), and some of these developments will be highlighted below. The discoveries of such epigenetic changes have ignited a revolution not only in the field of gene regulation but also in gene maintenance and gene evolution.

RNA-Dependent DNA Methylation

A role for RNA in guiding de novo cytosine methylation of homologous DNA sequences was first discovered in viroid-infected plants and subsequently also in nonpathogenic plant systems (194). When the dsRNA degradation mediated PTGS occurs in plants, the genomic DNA regions homologous to dsRNA are often found methylated at almost all the sensitive cytosine residues. This process is generally referred to as RNA-dependent-DNA methylation and the corresponding part of the genome, especially the promoter region might remain transcriptionally silent. The initiator of RNA-dependent DNA methylation/TGS could be either the transgene-derived dsRNA or the consequent siRNA (110, 111, 214). Depending on the sequence information of the dsRNA, RNA-dependent DNA methylation was found to occur at the open reading frame and/or the promoter region of the genome (10, 149). If methylation occurred only at the open reading frame, TGS did not result. However, RNA-dependent DNA methylation at the promoter sequences induced TGS, which, unlike PTGS, was stable and heritable (98). RNA-dependent DNA methylation within the host genes has also been found to occur preponderantly during virus-induced gene silencing, a type of RNAi that is generally initiated by plant virus vectors carrying portions of host genes, as has been described earlier (214).

It was demonstrated that the movement of transposons was controlled by transcriptional suppression (TGS) and that methylation also played a role in this suppression, depending on the nature of the transposon (226). In animals and lower plants, siRNAs corresponding to the transposable elements were discovered and cloned earlier (9, 232), and in *A. thaliana* and *Nicotiana* species, the siRNAs corresponding to retroelements have recently been discovered (94). These siRNAs are perhaps responsible for the methylation of the homologous DNA.

There are also conflicting data in the literature concerning the cause-and-effect relationship between PTGS and DNA methylation. In some examples, there is no correlation between PTGS and DNA methylation (153). In other events, as mentioned earlier, the correlation is strong (137). Llave et al. (137) showed that a viral protein, HC-PRO, that suppresses PTGS/RNAi, when introduced into GUS-silenced tobacco, inhibited the maintenance of small RNAs and caused a concomitant decrease in methylation of the GUS sequence in the plant genome. This study suggested that DNA methylation of the silenced gene could be directly correlated with PTGS. However, in a contrasting study carried out by Mette et al. (153), HC-PRO was found to increase the methylation of a target promoter DNA when gene silencing was induced by the promoter dsRNA. The later study also revealed that the amount of promoter siRNA was elevated fivefold in the presence of HC-PRO. Taken together, both of these studies indicate that the level of target DNA methylation is directly related to the amount of siRNA present in the cell, and thus the apparent differences between these observations can be resolved. In other words, the availability of siRNA may determine the level of RNA-directed DNA methylation. In the events of RNA-dependent DNA methylation, the chromodomain containing DNA methylases acts either alone or in combination with other proteins, such as piwi-containing proteins, to form complexes with the siRNAs and cause sequence-specific RNA-dependent DNA methylation, finally resulting in TGS (10).

Evidence of cross talk between PTGS and TGS has been obtained from the mutational analysis of *A. thaliana* and *D. melanogaster*. Two types of *A. thaliana* mutants, *ddm1* (deficient in DNA methylation) and *met1* (methyl transferase), were isolated from a screen of mutations causing a reduction in global methylation of the genome. The locus *ddm1* encodes an SNF2/SW12-like chromatin-modeling protein, whereas MET1 is a major DNA methyltransferase. Both of these mutants exhibit marked reduction in PTGS activity, as measured by the accumulation of transgene transcripts (10, 218). Although the patterns of reduction are different with these mutants, these studies highlight the strong correlation between PTGS and TGS.

In *D. melanogaster*, polycomb protein-dependent TGS is also affected by mutations in PIWI, a family of proteins required for RNAi (169). Other evidence includes the *argonaute4* gene of *A. thaliana*, which controls both locus-specific siRNA accumulation and DNA methylation (241); the *Arabidopsis sde4* locus, which is of unknown biochemical function but is responsible for (retroelement TS SINE-specific) siRNA formation (94); and the *Arabidopsis rts1* (RNA-mediated transcription silencing) mutation, which causes a ≈50% reduction in target promoter DNA methylation (10). However, not all TGS mutations affect the PTGS pathways and vice versa, suggesting that the two pathways diverge at some point (218).

RNA-dependent DNA methylation has been reported only in plants until now. Aufsatz et al. (10) have also shown that

asymmetric non-CpG methylation is mostly affected by RNA-dependent DNA methylation, but the existence of non-CpG methylation in mammals has always been a contentious issue. Mammalian DNA is methylated mostly at symmetric CpG or CNG sites by various forms of DNA methyltransferases. However, using a dual-labeling nearest-neighbor technique and the bisulfite genomic sequencing methods, Ramsahoye et al. (177) found that the genomes of embryonic stem cells but not that of somatic tissues harbored non-CpG methylation, which accounted for 15 to 20% of total cytosine methylation. This methylation is perhaps caused by the methylase Dnmt 3a, which is highly expressed in embryonic stem cells but poorly expressed in somatic tissues (179). Other studies have also revealed that in *D. melanogaster* and mammals, non-CpG methylation is an early embryonic event (10), and this methylation can be catalyzed by Dnmt 2, which is primarily active at the initial stages of development (142).

In the above-mentioned studies, however, no connection between non-CpG methylation and any homologous RNA has been shown. Hence, if RNA-dependent DNA methylation occurs at all in animals, it might be limited to the early developmental stages when the effector proteins may be found in abundance. In contrast, RNA-dependent DNA methylation is observed throughout plant development, implying the continuous availability of the appropriate plant DNA methyltransferases. This feature also explains the ease of RNA-dependent DNA methylation detection in plants (10).

Heterochromatin Formation

Even for organisms in which RNA-dependent DNA methylation is supposedly absent, there is growing evidence that RNAi processes cause chromatin modifications leading to TGS. This evidence reveals that the connections between TGS and PTGS are strong across all layers of eukaryotic life. For example, in *C. elegans*, in which DNA methylation has not been detected, some PTGS mutations, namely *mut7* and *rde2*, derepress transgenes which are affected by polycomb-dependent TGS (203). The polycomb group of proteins are known to keep the chromatin in the closed or compact conformation. Conversely, it has also been found recently that the polycomb proteins MES3, MES4, and MES6 are required for RNAi, at least under some experimental conditions (65, 121).

Generally, in eukaryotic systems, histone modifications make the chromatin structure inert to transcription by heterochromatin formation, which is modulated greatly by the RNAi processes, as recent discoveries have revealed. In almost all organisms heterochromatin formation requires that histone H3 of the chromatin be deacetylated and then methylated at lysine 9. The SET domain of a special group of histone methyltransferases carries out this function. This methylated lysine is subsequently bound by a heterochromatin binding protein, HP1. The binding of the chromodomain containing HP1 to Met H3-K9 is highly specific and of very high affinity (12). This binding may be followed by multimerization of HP1 and complex formation with other chromatin-remodeling proteins. As a result of this multicomplex formation, the chromatin becomes condensed and locked in a transcriptionally repressed heterochromatic state.

Once formed, the heterochromatin spreads a large distance due to cooperative protein-protein interactions of chromatin-remodeling factors, the components of which have not been fully identified yet. However, these structures are generally initiated at places containing repeated DNA sequences, for example, centromere, telomere, mating locus, and elsewhere in the genome containing repetitive DNA in the fission yeast *Schizosaccharomyces pombe* (7). These repeats are responsible for producing dsRNAs, which are processed by the RNAi machinery. D. P. Bartel's group has discovered abundant species of centromeric repeat-specific siRNAs from *S. pombe* (180). Volpe et al. (223) demonstrated that these siRNAs are blocked and instead, large noncoding RNAs (≈1.4 to 2.4 kb) homologous to the centromere repeats accumulate in *dcr1*, *ago1*, and *rdrp1* mutants of *S. pombe*. These mutant cells also do not show the heterochromatin-mediated silencing of a *ura4*$^+$ gene inserted into the outer and inner repeats that flank the central core of the centromeres. A corresponding reduction in Met-H3 K9 is also observed in the outer repeats of these mutant cells (223). This loss in gene silencing is phenotypically similar in cells lacking the histone methyltransferase (*clr4*) or the HP1-like (*swi6*) activity.

That the DNA repeats are central to the RNAi-like processing of dsRNA and concomitant heterochromatin formation was clearly established by the findings of Hall and colleagues (89), who inserted a 3.6-kb centromere H repeat, normally present at the silent mating type domain, in a euchromatic position (*ura4* locus). The introduction of this repeat was sufficient to turn on the silencing of a linked reporter gene and induce H3-K9 methylation and recruitment of HP1-like factors (Swi6) (89). The link between the RNAi machinery and heterochromatin formation has also been established by a recent finding in *A. thaliana*. From a large screen of mutants, Zilberman et al. (241) found that the *ago4* gene is responsible for the RNAi-related silencing of the *A. thaliana superman* gene, which is implicated in flower formation. The *ago4-1* mutation reactivates the silent *superman* allele and decreases non-CpG as well as H3-K9 methylation. Significantly, the same mutation also blocks DNA methylation and the accumulation of siRNA corresponding to the retroelement at SN1 (241).

The above-mentioned facts are put together in a model (Fig. 3) showing a link between siRNA and heterochromatin formation. In the wild-type scenario, one strand of the centromeric region is constitutively expressed, whereas the complementary strand, which is subjected to heterochromatic repression, is occasionally transcribed (57). Such transcription will lead to the formation of dsRNA, which will be processed by the RNAi machinery. This processing might even be a nuclear step, since a component of this machinery, the RdRP, was found to be physically bound to the outer repeats of the centromeric region in a chromatin immunoprecipitation assay (223). The siRNA thus formed might enter a complex containing the histone methyltransferase enzyme. This complex could be a nuclear equivalent of the RISC complex (Nu.RISC of Fig. 3) lacking nuclease activity (98). Such a complex would be guided to the appropriate DNA region following the DNA-RNA base pairing rules, and the histone H3-K9 of the region might be methylated to eventually generate the heterochromatin structure. Since RdRP is found locally, the spread of the heterochromatic structure may be associated with the extension of the 3′ end of the siRNA primer. It has also been shown in *N. crassa* and *A.*

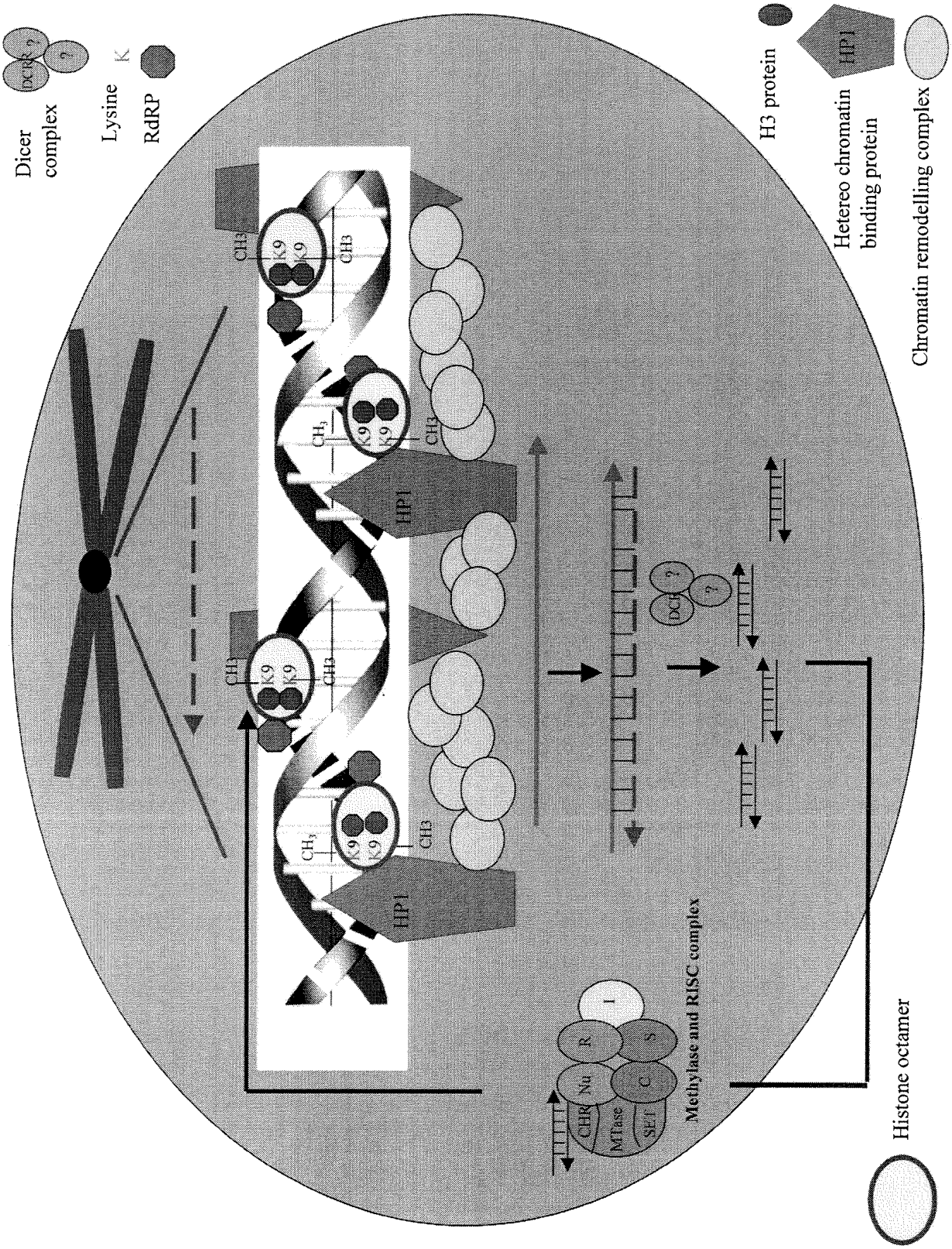

FIG. 3. Role of the RNAi process in heterochromatinization of nuclear DNA. The methylated (H3-K9) histone and many chromatin components are involved in the cross talk in several epigenetic regulatory pathways within the nucleus. The centromeric region (black oval) of the chromatin (thick purple line) might be responsible for the production of dsRNA transcripts (continuous red line and broken blue line). The transcript coming out of the DNA strand subjected to heterochromatinization is represented by broken blue lines. The siRNAs of the nucleus join the complex, of which the histone methyltransferase is a constituent. The siRNA binding the chromodomain (CHR), methyltransferase (MTase), and the SET domain of the methyltransferase are indicated.

thaliana that H3-K9 methylation directs DNA methylation (107, 204). The methylated DNA could be complexed further with the methyl-binding proteins. Following these binding events, the chromatin structure will be extremely compact and condensed and would remain transcriptionally inert.

DNA Elimination

The most dramatic effect of siRNA-mediated heterochromatin formation followed by chromosomal DNA elimination and rearrangement has been recorded in the ciliated protozoan *Tetrahymena pyriformis* (156, 206). Among unicellular organisms, *T. pyriformis* is unique because of its nuclear dimorphism. The two nuclei, the micronucleus and macronucleus, serve different functions. The polyploid macronucleus is the transcription center of the cell during vegetative growth, whereas the diploid and transcriptionally inert micronucleus acts as the germ line nucleus. During conjugation, the micronucleus gives rise to the macronucleus, and this transition is accompanied by two interesting and peculiar recombinant events. First, approximately 6,000 internal eliminated sequences of five pairs of micronucleus chromosomes, accounting for about 15% of genomic micro-DNA, are removed. Second, the remaining parts of these chromosomes are broken into 200 to 300 minichromosomes concomitant with the deletion of <50 nucleotide breakage eliminated sequences. The mechanisms of removal of internal eliminated sequences and breakage eliminated sequences remained elusive for a long time but were recently unveiled, courtesy of the awareness of the siRNA world.

Mochizuki et al. (156) showed that in wild-type cells of *Tetrahymena pyriformis*, siRNAs of about ≈26 to ≈31 nucleotides were produced which hybridized to micronuclear genomic DNA and not the macronuclear DNA, indicating that these siRNAs could be internal eliminated sequence/breakage eliminated sequence-specific and are referred to as scan RNAs. These scan RNAs were not made in *twi1* mutants, and the production of internal eliminated sequence/breakage eliminated sequence elements was also impaired by the *twi1* mutation. *twi1* produces a Piwi-related protein during the sexual cycle and can transmit the RNA-encoded information from the micronucleus to the old macronucleus and finally to the new macronucleus to mark the sequences to be eliminated (57). The defect in accumulation of the scan RNAs in the *twi1* mutant was similar to the case of another mutant, *pdd1* (156). In a related report, Taverna et al. (206) showed that the protein PDD1 was the effector protein for DNA excision and that PDD1 along with Met H3-K9 was associated preferentially with the internal eliminated sequence/breakage eliminated sequence elements in the new macronucleus that developed from the micronucleus during the sexual cycle. PDD1 contains two chromodomains and an additional RNA-binding domain (3).

The above data and the model presented in the earlier section lead to a straightforward and interesting scheme for programmed DNA degradation in *Tetrahymena pyriformis*. The bidirectional transcription that occurs across the internal eliminated sequence repeats (38) may form the dsRNA, which would give rise to the scan RNAs following the action of RNAi-related Dicing complexes that perhaps also include the Twi1 and PDD proteins. These scan RNAs eventually may be associated with the nuclear equivalents of RISC factor in the new macronucleus to provide heterochromatic sites at the internal eliminated sequence/breakage eliminated sequence regions. The chromodomain containing PDD proteins may remain bound to the scan RNA and thus guide to destroying the cognate DNA. As an extension of this work, Yao et al. found that a similar RNAi process recognized and deleted a foreign neomycin resistance gene of bacterial origin which was integrated in a *Tetrahymena* chromosome (237). These two studies together strongly suggest an siRNA- (or scan-RNA)-based mechanism that controls genome-wide DNA arrangements and provides genomic surveillance against invading foreign DNAs.

Thus, the *Tetrahymena pyriformis* as well as *S. pombe* data show how dramatic the epigenetic consequences of the genome could be following the formation of siRNA molecules in cells. Discovery of the link between the RNAi processes and the epigenetic chromatin modification as well as chromosome behavior is probably the most fascinating and novel face of regulation of gene silencing mechanism. The RNAi machinery is reported to control many explosive features of cellular biology, namely stem cell maintenance (53), cell fate determination (21), nonrandom chromosome segregation (188), etc. A recent report established that the fission yeast RNAi-related genes *ago1*, *dcr1*, and *rdp1* also control the fidelity of chromosome segregation during mitosis and meiosis. As discussed earlier, these gene products are required to maintain centromeric silencing. The report also demonstrated that the chromosome missegregation of the RNAi mutants occurred due to the loss of centromeric cohesion, suggesting a clear link between centromeric silencing and cohesion. This report broadly hinted that the regulation of chromosomal dynamics could be largely traced to the natural RNAi biology of the eukaryotic cells (90).

It is not difficult to imagine that we might witness RNAi-related unifying signals in diverse chromosome behaviors, namely X-chromosome inactivation, satellite-repeat contraction and expansion, hybrid dysgenesis in *D. melanogaster*, chromatin diminution in ascarid nematodes, nuclear dominance in plants, and so on in the not so distant future (57).

APPLICATIONS OF RNAi

Besides being an area of intense, upfront basic research, the RNAi process holds the key to future technological applications. Genome sequencing projects generate a wealth of information. However, the ultimate goal of such projects is to accelerate the identification of the biological function of genes. The functions of genes can be analyzed with an appropriate assay, by examining the phenotype of organisms that contain mutations in the gene, or on the basis of knowledge gained from the study of related genes in other organisms. However, a significant fraction of genes identified by the sequencing projects are new and cannot be rapidly assigned functions by these conventional methods.

RNAi technology is proving to be useful to analyze quickly the functions of a number of genes in a wide variety of organisms. RNAi has been adapted with high-throughput screening formats in *C. elegans*, for which the recombination-based gene knockout technique has not been established. Chromosomes I

and III of *C. elegans* have been screened by RNAi to identify the genes involved in cell division and embryonic development (82, 84). Recently, a large-scale functional analysis of ≈19,427 predicted genes of *C. elegans* was carried out with RNA interference. This study identified mutant phenotypes for 1,722 genes (112). Similarly, in *D. melanogaster*, RNAi technology has been successfully applied to identify genes with essential roles in biochemical signaling cascades, embryonic development, and other basic cellular process (44). In plants, gene knockdown-related functional studies are being carried out efficiently when transgenes are present in the form of hairpin (or RNAi) constructs. Plant endotoxins could also be removed if the toxin biosynthesis genes are targeted with the RNAi constructs. Recently, the theobromine synthase of the coffee plant was knocked down with the hairpin construct of the transgene, leading to the production of decaffeinated coffee plants (166). Virus-induced gene silencing has also been proven to be a successful approach for plant genetics (15).

Given the fact that RNAi is easy to apply, whole-genome screens by RNAi may become a common method of choice in the near future. RNAi may facilitate drug screening and development by identifying genes that can confer drug resistance or genes whose mutant phenotypes are ameliorated by drug treatment, providing information about the modes of action of novel compounds. Although RNAi is unlikely to replace the existing knockout technology, it may have a tremendous impact for those organisms that are not amenable to the knockout strategy. It may also be a method of choice to study the simultaneous functions of a number of analogous genes in organisms in which redundancy exists with respect to a particular function, because many of these genes can be silenced simultaneously.

Given the gene-specific features of RNAi, it is conceivable that this method will play an important role in therapeutic applications. Since siRNAs direct cellular RNAi biology, these are potential therapeutic reagents because of their power to downregulate the expression pattern of mutant genes in diseased cells. However, central to this hypothesis is the assumption that the effect of exogenous siRNA applications will remain gene specific and show no nonspecific side effects relating to mismatched off-target hybridization, protein binding to nucleic acids, etc. Though it was demonstrated that mismatches of more than even one nucleotide within the 19- to 20-mer siRNAs effectively disrupted proper degradation of the target mRNA (68), the gene specificity of siRNAs needs to be confirmed on a genome-wide scale.

Recently, Chi et al. (41) reported that the GFP siRNA-induced gene silencing of transient or stably expressed GFP mRNA was highly specific in the human embryonic kidney (HEK) 293 cell background. The specific silencing did not produce secondary changes in global gene expression, as detected by the DNA microarray experiment. They also failed to detect the presence of transitive RNAi in experimentally engineered human cell lines (41). In their own experiments, Semizarov et al. (190) reached a similar conclusion while using siRNAs corresponding to *akt1*, *rb1*, and *plk1* in the human non-small cell lung carcinoma cell line H1299. These experiments prove that siRNAs could be used as highly specific tools for targeted gene knockdown and can be used in high-throughput approaches and drug target validation. This exquisite sequence-specific effect of siRNAs has also been exploited in silencing the mutant allele of the diseased gene while not affecting the wild-type allele of the healthy version of the same gene (158).

siRNAs have been shown to inhibit infection by human immunodeficiency virus, poliovirus, and hepatitis C virus in cultured cell lines (152). Bitko and Barik (19) successfully used siRNAs to silence genes expressed from respiratory syncytial virus, an RNA virus that causes severe respiratory disease in neonates and infants. siRNA treatment has also been shown to reduce the expression of the BCR-ABL oncoprotein in leukemia and lymphoma cell lines, leading to apoptosis in these cells (230). With respect to future medical applications, siRNA-based therapy seems to have a great potential to combat carcinomas, myeloma, and cancer caused by overexpression of an oncoprotein or generation of an oncoprotein by chromosomal translocation and point mutations (211).

Recently, the therapeutic potential of the siRNA technique has been demonstrated in vivo in mouse models. McCaffrey et al. (151) and Song et al. (199) demonstrated effective targeting of a sequence from hepatitis C virus and the *fas* gene by RNA interference in mouse liver (199). An epiallelic series of p53 hypomorphs created by RNAi have been shown to produce distinct tumor phenotypes in mice in vivo, suggesting that RNAi can stably suppress gene expression (99). Song et al. (199) have shown that treatment with *fas* siRNA abrogated hepatocyte necrosis and inflammatory infiltration and protected mice from liver fibrosis and fulminant hepatitis. Rubinson et al. (184) showed highly specific, stable, and functional silencing of gene expression in transgenic mice with the lentivirus system for the delivery of siRNAs.

Although the delivery of siRNAs to a proper site remains problematic for gene therapy, chemical modifications of siRNAs such as changing the lipophilicity of the molecules or the methods previously developed for the application of antisense oligonucleotides or nuclease-resistant ribozymes might help the entry and stability of siRNAs within the transfected cells or tissues. The absence of specific micro-RNAs has been demonstrated in carcinoma cells, implying that cancer development could be arrested by introduction of the missing micro-RNAs. The micro-RNAs could be supplied in the form of siRNAs, since the function of micro-RNAs can be mimicked by the exogenous siRNA (62). However, independent of its biomedical applications, RNAi appears to be a forthcoming method for functional genomics.

CONCLUDING REMARKS

In the footsteps of the discovery of the double-helical structure of DNA, some outstanding discoveries have been recorded, but few of them really match the explosive content and implication of dsRNA-mediated gene silencing. This homology-dependent silencing has established a novel paradigm with far-reaching consequences in the field of transcription regulation. The regulatory mechanism offers cellular protection against parasitic nucleic acid sequences, carries out epigenetic as well as genetic alterations on the one hand, and governs organisms architecture and development on the other. Capitalizing on the basic principles of silencing, large-scale functional genomics have come into play in diverse organisms.

Studies conducted at the laboratory level have revealed the tremendous power of siRNAs as therapeutics and have demonstrated the potential of micro-RNAs to reverse cellular developmental aberrations.

The new paradigm has a lot more to offer than it has delivered already. The stepwise detailed mechanism of RNAi and its related processes is waiting to be explored. The rationale for many unexplained genetic findings of RNAi in worms, plants, and other organisms will be revealed in the wake of further mechanistic discoveries. The cytoplasmic location of RNAi is evident, but the evidence of nuclear connections of RNAi and related events are also too many. Surprisingly, there are some components of RNAi, GEMIN3 and GEMIN4 of humans, which partition in both the nuclear and cytoplasmic compartments. Hence, clarification of the subcellular locations of the RNAi processes is required. Hopefully, the detailed biochemical framework of RNAi would provide such clarifications.

As we gain more insight into the mechanisms, more effective methods for analysis of gene functions may evolve. We may learn more about geriatrics, nervous diseases, genetic imprints, nuclear dominance in plants, and so on and thus might wield control over such processes in the future. Meanwhile, the knockdown technology might improve vastly with better-designed plasmid- or virus-based vectors for delivery of siRNAs to the appropriate tissues at the appropriate time. Such technology is bound to give a new shape to therapeutic gene silencing as well. The science and technology of RNAi has given us a cultural ocean of virtually bottomless depth.

REFERENCES

1. **Aboobaker, A. A., and M. L. Blaxter.** 2003. Use of RNA interference to investigate gene function in the human filarial nematode parasite *Brugia malayi*. Mol. Biochem. Parasitol. **129:**41–51.
2. **Abraham, N., D. J. Stojdl, P. L. Duncan, N. Methot, T. Ishii, M. Dube, B. C. Vanderhyden, H. L. Atkins, D. A. Gray, and M. W. McBurney.** 1999. Characterization of transgenic mice with targeted disruption of the catalytic domain of the double-stranded RNA-dependent protein kinase, PKR. J. Biol. Chem. **274:**5953–5962.
3. **Akhtar, A., D. Zinc, and P. B. Becker.** 2000. Chromodomains are protein-RNA interaction modules. Nature **407:**405.
4. **Al-Anouti, F., T. Quach, and S. Ananvoranich.** 2003. Double-stranded RNA can mediate the suppression of uracil phosphoribosyltransferase expression in *Toxoplasma gondii*. Biochem. Biophys. Res. Commun. **302:**316–323.
5. **Al-Kaff, N. S., S. N. Covey, M. M. Kreike, A. M. Page, R. Pinder, and P. Dale.** 1998. Transcriptional and posttranscriptional plant gene silencing in response to a pathogen. Science **279:**2113–2115.
6. **Allas, U., A. Liiv, and J. Remme.** 2003. Functional interaction between RNase III and the Escherichia coli ribosome. BMC Mol. Biol. **4:**8–15.
7. **Allshire, R.** 2002. Molecular biology. RNAi and heterochromatin—a hushed-up affair. Science **297:**1818–1819.
8. **Anandalakshmi, R., G. J. Pruss, X. Ge, R. Marathe, A. C. Mallory, T. H. Smith, and V. B. Vance.** 1998. A viral suppressor of gene silencing in plants. Proc. Natl. Acad. Sci. USA **95:**13079–13084.
9. **Aravin, A. A., N. M. Naumova, A. V. Tulin, V. V. Vagin, Y. M. Rozovsky, and V. A. Gvozdev.** 2001. Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the *D. melanogaster* germline. Curr. Biol. **11:**1017–1027.
10. **Aufsatz, W., M. F. Mette, J. Van der Winden, A. J. Matzke, and M. Matzke.** 2002. RNA-directed DNA methylation in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **99:**16499–16506.
11. **Baehrecke, E. H.** 2003. miRNAs: Micro managers of programmed cell death. Curr. Biol. **13:**R473–R475.
12. **Bannister, A. J., P. Zegerman, J. F. Partridge, E. A. Miska, J. O. Thomas, R. C. Allshire, and T. Kouzarides.** 2001. Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature **410:**120–124.
13. **Bass, B. L.** 2000. Double-stranded RNA as a template for gene silencing. Cell **101:**235–238.
14. **Bastin, P., A. Galvani, and L. Sperling.** 2001. Genetic interference in protozoa. Res. Microbiol. **302:**316–323.
15. **Baulcombe, D. C.** 2001. Fast forward genetics based on virus induced gene silencing. Curr. Opin. Plant Biol. **2:**109–113.
16. **Bender, J.** 2001. A vicious cycle: RNA silencing and DNA methylation in plants. Cell **106:**129–132.
17. **Bernstein, E., A. A. Caudy, S. M. Hammond, and G. J. Hannon.** 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature **409:**363–366.
18. **Bingham, P. M.** 1997. Cosuppression comes to the animals. Cell **90:**385–387.
19. **Bitko, V., and S. Barik.** 2001. Phenotypic silencing of cytoplasmic genes with sequence specific double-stranded short interfering RNA and its application in the reverse genetics of wild-type negative strand RNA virus. BMC Microbiol. **1:**34–44.
20. **Blaszczyk, J., J. E. Trppea, M. Bubunenko, K. M. Routzahn, D. S. Waugh, D. L. Court, and X. Ji.** 2001. Crystallographic and modeling studies of RNase III suggest a mechanism for double-stranded RNA cleavage. Structure **9:**1225–1236.
21. **Bohmert, K., I. Camus, C. Bellini, D. Bouchez, M. Caboche, and C. Benning.** 1998. AGO 1 defines a novel class of *Arabidopsis* controlling leaf development. EMBO J. **17:**170–180.
22. **Boutet, S., F. Vazquez, J. Liu, C. Beclin, M. Fagard, A. Gratias, J. B. Morel, P. Crete, X. Chen, and H. Vaucheret.** 2003. *Arabidopsis* HEN1. A genetic link between endogenous miRNA controlling development and siRNA controlling transgene silencing and virus resistance. Curr. Biol. **13:**843–848.
23. **Boyle, J. P., X. J. Wu, C. B. Shoemaker, and T. P. Yoshino.** 2003. With RNA interference to manipulate endogenous gene expression in *Schistosoma mansoni* sporocysts. Mol. Biochem. Parasitol. **128:**205–215.
24. **Brennecke, J., D. R. Hipfner, A. Stark, R. B. Russell, and S. M. Cohen.** 2003. bantam encodes a developmentally regulated micro-RNA that controls cell proliferation and regulates the proapoptotic gene hid in *Drosophila*. Cell **113:**25–36.
25. **Brigneti, G., O. Voinnet, W. X. Li, L. H. Ji, S. W. Ding, and D. C. Baulcombe.** 1998. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. EMBO J. **17:**6739–6746.
26. **Brooks, D. R., and R. E. Isaac.** 2002. Functional genomics of parasitic worms: the dawn of a new era. Parasitol. Int. **51:**319–325.
27. **Brown, S. J., J. P. Mahaffey, M. D. Lorenzen, R. E. Denell, and J. W. Mahaffey.** 1999. With RNAi to investigate orthologous homeotic gene function during the development of distantly related insects. Evol. Dev. **1:**11–15.
28. **Brummelkamp, T. R., R. Bernards, and R. Agami.** 2002. A system for stable expression of short interfering RNAs in mammalian cells. Science **296:**550–553.
29. **Cali, B. M., S. L. Kuchma, J. Latham, and P. Anderson.** 1999. Smg-7 is required for mRNA surveillance in *C. elegans*. Genetics **151:**605–616.
30. **Calin, C. A., D. Dumitru, M. Shimizu, R. Bichi, S. Zupo, S. Noch, H. Aldler, S. Rattan, M. Keating, K. Rai, L. Rassenti, T. Kipps, M. Negrini, F. Bullrich, and C. M. Croce.** 2002. Frequent deletions and downregulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc. Natl. Acad. Sci. USA **99:**15524–15529.
31. **Caplen, N. J., S. Parrish, F. Imani, A. Fire, and R. A. Morgan.** 2001. Specific inhibition of gene expression by small double-stranded RNA in invertebrate and vertebrate systems. Proc. Natl. Acad. Sci. USA **98:**9742–9747.
32. **Carmell, M. A., Z. Xuan, M. Q. Zhang, and G. J. Hannon.** 2002. The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. Genes Dev. **16:**2733–2742.
33. **Carrington, J. C., and V. Ambros.** 2003. Role of micro-RNAs in plant and animal development. Science **301:**336–338.
34. **Catalanotto, C., G. Azzalin, G. Macino, and C. Cogoni.** 2000. Gene silencing in worms and fungi. Nature **404:**245.
35. **Caudy, A. A., M. Myers, G. J. Hannon, and S. M. Hammond.** 2002. Fragile X-related protein and VIG associate with RNA interference machinery. Genes Dev. **16:**2491–2496.
36. **Cerruti, L., N. Mian, and A. Bateman.** 2000. Domains in gene silencing in cell differentiation proteins: the novel PAZ domain and redefinition of the Piwi domain. Trends Biochem. Sci. **25:**481–482.
37. **Cerutti, H.** 2003. RNA interference: traveling in the cell and gaining functions? Trends Genet. **19:**39–46.
38. **Chalker, D. L., and M. C. Yao.** 2001. Nongenic, bidirectional transcription precedes and may promote developmental DNA deletion in *Tetrahymena thermophila*. Genes Dev. **15:**1287–1298.
39. **Chen, X.** 11 September 2003. A micro-RNA as a translational repressor of APETALA2 in *Arabidopsis* flower development. Science 10.1126/Science.1088060.
40. **Chen, X., J. Liu, Y. Cheng, and D. Jia.** 2002. HEN1 functions pleiotropically in *Arabidopsis* development and acts in C function in the flower. Development **129:**1085–1094.
41. **Chi, J. T., H. Y. Chang, N. N. Wang, D. S. Chang, N. Dunthy, and P. O. Brown.** 2003. Genomewide view of gene silencing by small interfering RNAs. Proc. Natl. Acad. Sci. USA **100:**6343–6346.
42. **Chiu, Y.-L., and T. M. Rana.** 2002. RNAi in human cells and functional features of small interfering RNA. Mol. Cell **10:**549–561.

43. **Chuang, C. F., and E. M. Meyerowitz.** 2000. Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **97**:4985–4990.
44. **Clemens, J. C., C. A. Worley, N. S. Leff, M. Merda, T. Maehama, B. A. Hemmings, and J. E. Dixon.** 2000. Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. Proc. Natl. Acad. Sci. USA **97**:6499–6503.
45. **Cogoni, C., and G. Macino.** 1997. Conservation of transgene-induced post-transcriptional gene silencing in plants and fungi. Trends Plant Sci. **2**:438–443.
46. **Cogoni, C., and G. Macino.** 1997. Isolation of quelling-defective (qde) mutants impaired in posttranscriptional transgene-induced gene silencing in *Neurospora crassa*. Proc. Natl. Acad. Sci. USA **94**:10233–10238.
47. **Cogoni, C., and G. Macino.** 1999. Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. Nature **399**:166–169.
48. **Cogoni, C., and G. Macino.** 1999. Posttranscriptional gene silencing in *Neurospora* by a RecQ DNA helicase. Science **286**:2342–2344.
49. **Cogoni, C., and G. Macino.** 2000. Post-transcriptional gene silencing across kingdoms. Curr. Opin. Genet. Dev. **10**:638–643.
50. **Cogoni, C., J. T. Irelan, M. Schumacher, T. J. Schmidhauser, E. U. Selker, and G. Macino.** 1996. Transgene silencing of al-1 gene in vegetative cells of *Neurospora* is mediated by a cytoplasmic effector and does not depend on DNA-DNA interaction or DNA methylation. EMBO J. **15**:3153–3163.
51. **Cogoni, C., N. Romano, and G. Macino.** 1994. Suppression of gene expression by homologous transgenes. Antonie Van Leeuwenhoek **65**:205–209.
52. **Cottrell, T. R., and T. L. Doering.** 2003. Silence of the strands: RNA interference in eukaryotic pathogens. Trends Microbiol. **11**:37–43.
53. **Cox, D. N., A. Chao, J. Baker, L. Chang, D. Qiao, and H. Lin.** 1998. A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. Genes Dev. **12**:3715–3727.
54. **Dalmay, T., A. Hamilton, S. Rudd, S. Angell, and D. C. Baulcombe.** 2000. An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. Cell **101**:543–553.
55. **Dalmay, T., R. Horsefield, T. H. Braunstein, and D. C. Baulcombe.** 2001. SDE3 encodes an RNA helicase required for post-transcriptional gene silencing in *Arabidopsis*. EMBO J. **20**:2069–2077.
56. **Depicker, A., and M. Van Montagu.** 1997. Post-transcriptional gene silencing in plants. Curr. Opin. Cell Biol. **9**:373–382.
57. **Dernburg, A. F., and G. H. Karpen.** 2002. A chromosome RNAissance. Cell **111**:159–162.
58. **Dernburg, A. F., J. Zalevsky, M. P. Colaiacovo, and A. M. Villeneuve.** 2000. Transgene–mediated cosuppression in the *C. elegans* germ line. Genes Dev. **14**:1578–1583.
59. **Devree, E., and P. A. Silver.** 2002. Retrovirus-delivered siRNA. BMC Biotechnol. **2**:15–19.
60. **Ding, S. W., B. J. Anderson, H. R. Hasse, and R. H. Symons.** 1994. New overlapping gene encoded by the cucumber mosaic virus genome. Virology **198**:593–601.
61. **Djikeng, A., H. Shi, C. Tschudi, and E. Ullu.** 2001. RNA interference in *Trypanosoma brucei*: cloning of small interfering RNA provides evidence for retroposon-derived 24–26 nucleotide RNA. RNA **7**:1522–1530.
62. **Doench, J. G., C. P. Petersen, and P. A. Sharp.** 2003. SiRNA can function as miRNA. Genes Dev. **17**:438–442.
63. **Domeier, M. E., D. P. Morse, S. W. Knight, M. Portereiko, B. L. Bass, and S. E. Mango.** 2000. A link between RNA interference and nonsense-mediated decay in *Caenorhabditis elegans*. Science **289**:1928–1930.
64. **Donze, O., and D. Picard.** 2002. RNA interference in mammalian cells with siRNA synthesized with T7 RNA polymerase. Nucleic Acid Res. **30**:E46.
65. **Dudley, N. R., J. C. Labbe, and B. Goldstein.** 2002. With RNA interference to identify genes required for RNA interference. Proc. Natl. Acad. Sci. USA **99**:4191–4196.
66. **Dykxhoorn, D. M., C. D. Novina, and P. A. Sharp.** 2003. Killing the messenger: short RNAs that silence gene expression. Nat. Rev. Mol. Cell. Biol. **4**:457–467.
67. **Elbashir, S. M., J. Harborth, W. Lendecknel, A. Yalcin, K. Weber, and T. Tuschl.** 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in mammalian cell culture. Nature **411**:494–498.
68. **Elbashir, S. M., J. Martinez, A. Patkaniowska, W. Lendeckel, and T. Tuschl.** 2001. Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. EMBO J. **20**:6877–6888.
69. **Elbashir, S. M., W. Lendeckel, and T. Tuschl.** 2001. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. **15**:188–200.
70. **Elbashir, S. M., J. Harborth, K. Weber, and T. Tuschl.** 2002. Analysis of gene functions in somatic mammalian cells with small interfering RNAs. Methods **26**:199–213.
71. **English, J. J., E. Mueller, and D. C. Baulcombe.** 1996. Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. Plant Cell **8**:179–188.
72. **Escobar, M. A., E. L. Civerolo, K. R. Summerfelt, and A. M. Dandekar.** 2001. RNAi-mediated oncogene silencing confers resistance to crown gall tumorigenesis. Proc. Natl. Acad. Sci. USA **98**:13437–13442.
73. **Fagard, M., and H. Vaucheret.** 2000. (Trans) gene silencing in plants: how many mechanisms? Annu. Rev. Plant Physiol. Plant Mol. Biol. **51**:167–194.
74. **Fagard, M., and H. Vaucheret.** 2000. Systemic silencing signal(s). Plant Mol. Biol. **43**:285–293.
75. **Fagard, M., S. Boutet, J.-B. Morel, C. Bellini, and H. Vaucheret.** 2000. AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. Proc. Natl. Acad. Sci. USA **97**:11650–11654.
76. **Finnegan, E. J., R. Margis, and P. M. Waterhouse.** 2003. Posttranscriptional gene silencing is not compromised in the *Arabidopsis* CARPEL FACTORY (Dicer-LIKE1) mutant, a homolog of Dicer-1 from *Drosophila*. Curr. Biol. **13**:236–240.
77. **Fire, A.** 1999. RNA triggered gene silencing. Trends Genet. **15**:358–363.
78. **Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello.** 1998. Potent and specific genetic interference by double-stranded RNA in *C. elegans*. Nature **391**:806–811.
79. **Fjose, A., S. Ellingsen, A. Wargelius, and H. C. Seo.** 2001. RNA interference: mechanisms and applications. Biotechnol. Annu. Rev. **7**:31–57.
80. **Fortier, E., and J. M. Belote.** 2000. Temperature-dependent gene silencing by an expressed inverted repeat in *Drosophila*. Genesis **26**:240–244.
81. **Francesco, D. S., S. Hanspeter, L. Alejandro, T. Cornia, B. Estelle, and M. Frederick.** 2001. Sense and anti sense mediated gene silencing in tobacco is inhibited by the same viral suppressors and is associated with accumulation of small RNAs. Proc. Natl. Acad. Sci. USA **96**:6506–6510.
82. **Fraser, A. J. G., R. S. Kamath, P. Zipperten, M. M. Campos, M. Sohrmann, and J. Ahringer.** 2000. Functional genomic analysis of *C. elegans* chromosome I by systemic RNA interference. Nature **408**:325–330.
83. **Golden, T. A., S. E. Schauer, J. D. Lang, S. Pien, A. R. Mushegian, U. Grossniklaus, D. W. Meinke, and A. Ray.** 2002. SHORT INTEGUMENTS1/SUSPENSOR1/CARPEL FACTORY, a Dicer homolog, is a maternal effect gene required for embryo development in *Arabidopsis*. Plant Physiol. **130**:808–822.
84. **Gonczy, P., C. Echeverri, K. Oegema, A. Coulron, S. J. M. Jones, R. R. Copley, J. Duperon, J. Oegema, M. Brehm, E. Carrin, E. Hannak, M. Kirkham, S. Pichler, K. Flohrs, A. Gossen, S. Liedel, A. M. Alleaume, C. Martin, N. Ozlu, P. Bork, P., and A. A. Hyman.** 2000. Functional genomic analysis of cell division in *C. elegans* with RNAi of genes on chromosome III. Nature **408**:331–335.
85. **Grad, Y., J. Aach, G. D. Hayes, B. J. Reinhart, G. M. Church, G. Ruvkun, and J. Kim.** 2003. Computational and experimental identification of C. elegans micro-RNAs. Mol. Cell **11**:1253–1263.
86. **Grishok, A., A. E. Pasquinelli, D. Conte, N. Li, S. Parrish, I. Ha, D. L. Baillie, A. Fire, G. Ruvkun, and C. C. Mello.** 2001. Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell **106**:23–34.
87. **Grishok, A., H. Tabara, and C. C. Mello.** 2000. Genetic requirements for inheritance of RNAi in *C. elegans*. Science **287**:2494–2497.
88. **Guo, H.-S., J.-F. Fei, Qi-Xie, and N.-H. Chua.** 2003. A chemical-regulated inducible RNAi in plants. Plant J. **34**:383–392.
89. **Hall, I. M., G. D. Shankaranarayana, K.-I. Noma, N. Ayoub, A. Cohen, and S. L. Grewal.** 2002. Establishment and maintenance of a heterochromatin domain. Science **297**:2232–2237.
90. **Hall, I. M., K. Noma, and S. I. Grewal.** 2003. RNA interference machinery regulates chromosome dynamics during mitosis and meiosis in fission yeast. Proc. Natl. Acad. Sci. USA **100**:193–198.
91. **Hamann, L., K. Jensen, and K. Harbers.** 1993. Consecutive inactivation of both alleles of the gb110 gene has no effect on the proliferation and differentiation of mouse embryonic stem cells. Gene **126**:279–284.
92. **Hamilton, A. J., and D. C. Baulcombe.** 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science **286**:950–952.
93. **Hamilton, A. J., S. Brown, H. Yuanhai, M. Ishizuka, and A. Lowe.** 1998. A transgene with repeated DNA causes high frequency, post-transcriptional suppression of ACC-oxidase gene expression in tomato. Plant J. **15**:737–746.
94. **Hamilton, A., O. Voinnet, L. Chappell, and D. Baulcombe.** 2002. Two classes of short interfering RNA in RNA silencing. EMBO J. **21**:4671–4679.
95. **Hammond, S. M., A. A. Caudy, and G. J. Hannon.** 2001. Post-transcriptional gene silencing by double-stranded RNA. Nat. Rev. Genet. **2**:110–119.
96. **Hammond, S. M., E. Berstein, D. Beach, and G. J. Hannon.** 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature **404**:293–296.
97. **Hammond, S. M., S. Boettcher, A. A. Caudy, R. Kobayashi, and G. J. Hannon.** 2001. Argonaute2, a link between genetic and biochemical analyses of RNAi. Science **293**:1146–1150.
98. **Hannon, G. J.** 2002. RNA Interference. Nature **418**:244–251.
99. **Heman, M. T., J. S. Fridmann, J. T. Zilfou, E. Hernando, P. J. Paddison, C. Cordon-Cardo, G. J. Hannon, and S. W. Lowe.** 2003. An epi-allelic series of p53 hypomorphs created by stable RNAi produces distinct phenotypes *in vivo*. Nat. Genet. **33**:396–400.
100. **Hodgkin, J., A. Papp, R. Pulak, V. Ambros, and P. Anderson.** 1989. A new

kind of informational suppression in the nematode *Caenorhabditis elegans*. Genetics **123:**301–313.

101. **Holen, T., M. Amarzguioui, M. T. Wiiger, E. Babaie, and H. Prydz.** 2002. Positional effects of short interfering RNAs targetting the human coagulation trigger tissue factor. Nucleic Acid Res. **30:**1757–1766.
102. **Hughes, C. L., and T. C. Kaufman.** 2000. RNAi analysis of deformed, proboscipedia and sex combs reduced in the milkweed bug *Oncopeltus fasciatus*: novel roles for Hox genes in the hemipteran head. Development **127:**3683–3694.
103. **Hutvagner, G., and P. D. Zamore.** 2002. A micro-RNA in a multiple-turnover RNAi enzyme complex. Science **297:**2056–2060.
104. **Hutvagner, G., J. McLachlan, A. E. Pasquinelli, E. Balint, T. Tuschl, P. D. Zamore.** 2001. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science **293:**834–838.
105. **Ingelbrecht, I., H. Van Houdt, M. Van Montagu, and A. Depicker.** 1994. Post-transcriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. Proc. Natl. Acad. Sci. USA **91:**10502–10506.
106. **Ischizuka, A., M. C. Siomi, and H. Siomi.** 2002. A *Drosophila* fragile X protein interacts with components of RNAi and ribosomal proteins. Gene Dev. **16:**2497–2508.
107. **Jackson, J. P., A. M. Lindroth, X. Cao, and S. E. Jacobsen.** 2002. Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. Nature **416:**556–560.
108. **Jacobson, S. E., M. P. Running, and E. M. Meyerowitz.** 1999. Disruption of an RNA helicase/RNase III gene in *Arabidopsis* causes unregulated cell division in floral-meristems. Development **126:**5231–5243.
109. **Jenuwein, T.** 2002. Molecular biology. An RNA-guided pathway for the epigenome. Science **297:**2215–2218.
110. **Jones, L., A. J. Hamilton, O. Voinnet, C. L. Thomas, A. J. Maule, and D. C. Baulcombe.** 1999. RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. Plant Cell **11:**2291–2302.
111. **Jones, L., F. Ratcliff, and D. C. Baulcombe.** 2001. RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. Curr. Biol. **11:**747–757.
112. **Kamath, R. S., A. G. Fraser, Y. Dong, G. Poulin, R. Durbin, M. Gotta, A. Kanapin, N. I. Bot, S. Moreno, M. Sohrmann, D. P. Welchman, P. Zipperlen, and J. Ahringer.** 2003. Syst. functional analysis of the *Caenorhabditis elegans* genome with RNAi. Nature **421:**231–237.
113. **Kasschau, K. D., Z. Xie, E. Allen, C. Llave, E. J. Chapman, K. A. Krizan, and J. C. Carrington.** 2003. P1/HC-Pro, a viral suppressor of RNA silencing, interferes with *Arabidopsis* development and miRNA function. Dev. Cell **4:**205–217.
114. **Kennerdell, J. R., and R. W. Carthew.** 1998. Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. Cell **95:**1017–1026.
115. **Ketting, R. F., S. E. Fischer, E. Bernstein, T. Sijen, G. J. Hannon, and R. H. A. Plasterk.** 2001. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. Genes Dev. **15:**2654–2659.
116. **Ketting, R. F., T. H. A. Haverkamp, H. G. A. M. Van Luenen, and R. H. A. Plasterk.** 1999. *mut-7* of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNase D. Cell **99:**133–141.
117. **Kjemtrup, S., K. S. Sampson, C. G. Peele, L. V. Nguyen, M. A. Conkling, W. F. Thompson, and D. Robertson.** 1998. Gene silencing from plant DNA carried by geminivirus. Plant J. **14:**91–100.
118. **Klahre, U., P. Crete, S. A. Leuenberger, V. A. Iglesias, and F. Meins.** 2002. High molecular weight RNAs and small interfering RNAs induce systemic post-transcriptional gene silencing in plants. Proc. Natl. Acad. Sci. USA **99:**11981–11986.
119. **Knight, S. W., and B. L. Bass.** 2001. A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in *C. elegans*. Science **293:**2269–2271.
120. **Kooter, J. M., M. A. Matzke, and P. Meyer.** 1999. Listening to silent gene: transgene silencing, gene regulation and pathogen control. Trends Plant Sci. **4:**340–347.
121. **Korf, I., Y. Fan, and S. Strome.** 1998. The Polycomb group in *Caenorhabditis elegans* and maternal control of germline development. Development **125:**2468–2478.
122. **Korneev, S. A., I. Kemenes, V. Straub, K. Staras, E. I. Korneeva, G. Kemenes, P. R. Benjamin, and M. OShea.** 2002. Suppression of nitric oxide (NO)-dependent behavior by double-stranded RNA-mediated silencing of a neuronal NO synthase gene. J. Neurosci. **22:**RC227.
123. **Lagos-Quintana, M., R. Rauhut, W. Lendeckel, and T. Tuschl.** 2001. Identification of novel genes coding for small expressed RNAs. Science **294:**853–858.
124. **Lai, E. C.** 2002. Micro RNAs are complementary to 3′ untranslated region sequence motifs that mediate negative post-transcriptional regulation. Nat. Genet. **30:**363–364.
125. **Lau, N. C., L. P. Lim, E. G. Weinstein, and D. P. Bartel.** 2001. An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. Science **294:**858–862.
126. **Lee, R. C., and V. Ambros.** 2001. An extensive class of small RNAs in *Caenorhabditis elegans*. Science **294:**862–864.
127. **Lee, R. C., L. Feinbaum, and V. Ambros.** 1993. The *C. elegans* heterochronic Gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell **75:**843–854.
128. **Li, W. X., and S. W. Ding.** 2001. Viral suppressors of RNA silencing. Curr. Opin. Biotechnol. **12:**150–154.
129. **Lim, L. P., M. E. Glasner, S. Yekta, C. B. Burge, and D. P. Bartel.** 2003a. Vertebrate micro-RNA genes. Science **299:**1540.
130. **Lim, L. P., N. C. Lau, E. C. Weinstein, A. Abdelhakim, S. Yekta, M. W. Rhoades, C. B. Burge, and D. P. Bartel.** 2003b. The micro-RNAs of C. elegans. Genes Dev. **17:**991–1008.
131. **Reference deleted.**
132. **Lindbo, J. A., and W. G. Dougherty.** 1992. Untranslatable transcripts of tobacco etch virus coat protein gene sequence can interfere with tobacco etch virus replication in transgenic plants and protoplasts. Virology **189:**725–733.
133. **Lindbo, J. A., L. Silva-Rosales, W. M. Proebsting, and W. G. Dougherty.** 1993. Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resistance. Plant Cell **5:**1749–1759.
134. **Linder, L., and M.-C. Daugeron.** 2000. Are DEAD-box proteins becoming respectable helicases? Nat. Struct. Biol. **7:**97–99.
135. **Lipardi, C., Q. Wei, and B. M. Paterrson.** 2001. RNAi as random degradation PCR: siRNA primers convert mRNA into dsRNA that are degraded to generate new siRNAs. Cell **101:**297–307.
136. **Liu, Y., M. Schiff, G. Serion, X.-W. Deng, and S. P. Dinesh-kumar.** 2002. Role of SCF Ubiquitin-ligase and the Cop9 signalosome in the N-gene mediated resistance response to tobacco mosaic virus. Plant Cell **14:**1483–1496.
137. **Llave, C., K. D. Kasschau, and J. C. Carrington.** 2000. Virus encoded suppressor of post-transcriptional gene silencing targets a maintenance step in silencing pathway. Proc. Natl. Acad. Sci. USA **97:**13401–13406.
138. **Llave, C., K. D. Kasschau, M. A. Rector, and J. C. Carrington.** 2002. Endogenous and silencing-associated small RNAs in plants. Plant Cell **14:**1605–1619.
139. **Llave, C., Z. Xie, K. D. Kasschau, and J. C. Carrington.** 2002. Cleavage of Scarecrow-like mRNA targets directed by a class of *Arabidopsis* miRNA. Science **297:**2053–2056.
140. **Lohmann, J. U., I. Endl, and T. C. G. Bosch.** 1999. Silencing of developmental genes in hydra. Dev. Biol. **214:**211–214.
141. **Lucy, A. P., H. S. Guo, W. X. Li, and S. W. Ding.** 2000. Suppression of post transcriptional gene silencing by a plant viral protein localized in the nucleus. EMBO J. **19:**1672–1680.
142. **Lyko, F.** 2001. DNA methylation learns to fly. Trends Genet. **17:**169–172.
143. **Malhotra, P., P. V. N. Dasaradhi, A. Kumar, A. Mohmmed, N. Agrawal, R. K. Bhatnagar, and V. S. Chauhan.** 2002. Double-stranded RNA-mediated gene silencing of cysteine proteases (falcipain-1 and -2) of *Plasmodium falciparum*. Mol. Microbiol. **45:**1245–1254.
144. **Mallory, A. C., B. J. Reinhart, D. Bartel, V. B. Vance, and L. H. Bowman.** 2002. A viral suppressor of RNA silencing differentially regulates the accumulation of short interfering RNAs and micro-RNAs in tobacco. Proc. Natl. Acad. Sci. USA **99:**15228–15233.
145. **Mallory, A. C., L. Ely, T. H. Smith, R. Marathe, R. Anandalakshmi, M. Fagard, H. Vaucheret, G. Pruss, L. Bowman, and V. B. Vance.** 2001. HC-Pro suppression of gene silencing eliminates the small RNA but not the transgene methylation are the mobile signal. Plant Cell **13:**571–583.
146. **Marie, B., J. P. Bacon, and J. M. Blagburn.** 2000. Double-stranded RNA interference shows that Engrailed controls the synaptic specificity of identified sensory neurons. Curr. Biol. **10:**289–292.
147. **Martens, H., J. Novotny, J. Oberstrass, T. L. Steck, P. Postlethwait, and W. Nellen.** 2002. RNAi in Dictyostelium: the role of RNA-directed RNA polymerases and double-stranded RNase. Mol. Biol. Cell **13:**445–453.
148. **Mathews, D. H., J. Sabina, M. Zuker, and D. H. Turner.** 1999. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. J. Mol. Biol. **288:**911–940.
149. **Matzke, M., A. J. Matzke, and J. M. Kooter.** 2001. RNA: guiding gene silencing. Science **293:**1080–1083.
150. **McRobert, L., and G. A. McConkey.** 2002. RNA interference (RNAi) inhibits growth of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **119:**273–278.
151. **McCaffery, A. P., L. Meuse, T.-T. Pham, D. S. Conklin, G. J. Hannon, and M. A. Kay.** 2002. RNA interference in adult mice. Nature **418:**38–39.
152. **McManus, M. T., and P. A. Sharp.** 2002. Gene silencing in mammals by small interfering RNAs. Nat. Rev. Genet. **3:**737–747.
153. **Mette, M. F., A. J. Matzke, and M. A. Matzke.** 2001. Resistance of RNA-mediated TGS to He-Pro, a viral suppressor of PTGS, suggests alternate pathways for dsRNA processing. Curr. Biol. **11:**1119–1123.
154. **Metzlaff, M., M. ODell, P. D. Cluster, and R. B. Flavell.** 1997. RNA-mediated RNA degradation and chalcone synthase A silencing in *Petunia*. Cell **88:**845–854.
155. **Misquitta, L., and B. M. Paterson.** 1999. Targeted disruption of gene

function in *Drosophila* by RNA interference (RNA-i): a role for nautilus in embryonic somatic muscle formation. Proc. Natl. Acad. Sci. USA **96:**1451–1456.

156. **Mochizuki, K., N. A. Fine, T. Fujisawa, and M. A. Gorovsky.** 2002. Analysis of a piwi-related gene implicates small RNAs in genome rearrangement in *Tetrahymena*. Cell **110:**689–699.
157. **Morel, J. B., C. Godon, P. Mourrain, C. Beclin, S. Boutet, F. Feuerbach, F. Proux, and H. Vaucheret.** 2002. Fertile hypomorphic ARGONAUTE (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance. Plant Cell **14:**629–639.
158. **Moss, E. G.** 2003. Silencing unhealthy alleles naturally. Trends Biotechnol. **21:**185–187.
159. **Mourelatos, Z., J. Dostie, S. Paushkin, A. Sharma, B. Charroux, L. Abel, J. Rappsilber, M. Mann, and G. Dreyfuss.** 2002. miRNPs: a novel class of ribonucleoproteins containing numerous micro-RNAs. Genes Dev. **16:**720–728.
160. **Mourrain, P., C. Beclin, T. Elmayan, F. Feuerbach, C. Godon, J.-B. Morel, D. Jouette, A. M. Lacombe, S. Nikic, N. Picault, K. Remoue, M. Sanial, T.-A. Vo, and H. Vaucheret.** 2000. *Arabidopsis* SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. Cell **101:**533–542.
161. **Nakai, K., and P. Horton.** 1999. PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem. Sci. **24:**34–36.
162. **Nakano, H., S. Amemiya, K. Shiokawa, and M. Taira.** 2000. RNA interference for the organizer-specific gene Xlim-1 in *Xenopus* embryos. Biochem. Biophys. Res. Commun. **274:**434–439.
163. **Napoli, C., C. Lemieux, and R. Jorgensen.** 1990. Introduction of chimeric chalcone synthase gene into *Petunia* results in reversible cosuppression of homologous genes in trans. Plant Cell **2:**279–289.
164. **Nicholson, A. W.** 1999. Function, mechanism and regulation of bacterial ribonucleases. FEMS Microbiol. Rev. **23:**371–390.
165. **Nykanen, A., B. Haley, and P. D. Zamore.** 2001. ATP requirement and small interfering RNA structure in the RNA interference pathway. Cell **107:**309–321.
166. **Ogita, S., H. Uefuji, Y. Yamaguchi, N. Koizumi, and H. Sano.** 2003. Producing decaffeinated coffee plants. Nature **423:**823.
167. **Page, M. F., B. Carr, K. R. Anders, A. Grimson, and P. Anderson.** 1999. SMG-2 is a phosphorylated protein required for mRNA surveillance in *Caenorhabditis elegans* and related to Upf1p of yeast. Mol. Cell. Biol. **19:**5943–5951.
168. **Palauqui, J. C., T. Elmayan, J. M. Pollien, and H. Vaucheret.** 1997. Systemic acquired silencing: Transgene specific posttranscriptional gene silencing is transmitted by grafting from silenced stocks to nonsilenced scions. EMBO J. **16:**4738–4745.
169. **Pal-Bhadra, M., U. Bhadra, and J. A. Birchler.** 2002. RNAi-related mechanisms affect both transcriptional and posttranscriptional transgene silencing in *Drosophila*. Mol. Cell **9:**315–327.
170. **Pandolfini, T., B. Molesini, L. Avesani, A. Spena, and A. Polverari.** 2003. Expression of self-complementary hairpin RNA under the control of the rolC promoter confers systemic disease resistance to plum pox virus without preventing local infection. BMC Biotechnol. **3:**7–21.
171. **Park, W., J. Li, R. Song, J. Messing, and X. Chen.** 2002. CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in micro-RNA metabolism in Arabidopsis thaliana. Curr. Biol. **12:**1484–1495.
172. **Pasquinelli, A. E.** 2002. MicroRNAs: deviants no longer. Trends Genet. **18:**171–173.
173. **Peele, C., C. V. Jorden, N. Muangsan, M. Turnage, E. Egelkrout, P. Eagle, L. Hanely-Bowdoin, and D. Robertson.** 2001. Silencing of a-meristematic gene with geminivirus-derived vectors. Plant J. **27:**357–366.
174. **Piccin, A., A. Salameh, C. Benna, F. Sandrelli, G. Mazzotta, M. Zordan, E. Rosatol, C. P. Kyriacou, and R. Costa.** 2001. Efficient and heritable knockout of an adult phenotype in *Drosophila* with a GAL-4 driven hairpin RNA incorporating a heterologous spacer. Nucleic Acid Res. **29:**55–60.
175. **Provost, P., D. Dishart, J. Doucet, D. Frendewey, B. Samuelsson, and O. Radmark.** 2002. Ribonuclease activity and RNA binding of recombinant human Dicer. EMBO J. **21:**5864–5874.
176. **Rajagopal, R., S. Sivakumar, N. Agrawal, P. Malhotra, and R. K. Bhatnagar.** 2002. Silencing of midgut aminopeptidase N of *Spodoptera litura* by double-stranded RNA establishes its role as *Bacillus thuringiensis* toxin receptor. J. Biol. Chem. **277:**46849–46851.
177. **Ramsahoye, B. H., D. Biniszkiewicz, F. Lyko, V. Clark, A. P. Bird, and R. Jaenisch.** 2000. Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase *3a*. Proc. Natl. Acad. Sci. USA **97:**5237–5242.
178. **Raponi, M., and G. M. Arndt.** 2003. Double-stranded RNA-mediated gene silencing in fission yeast. Nucleic Acid Res. **31:**4481–4489.
179. **Ratcliff, F., B. D. Harrison, and D. C. Baulcombe.** 1997. A similarity between virus defense and gene silencing in plants. Science **276:**1558–1560.
180. **Reinhart, B. J., and D. P. Bartel.** 2002. Small RNAs correspond to centromere heterochromatic repeats. Science **297:**1831.
181. **Reinhart, B. J., E. G. Weinstein, M. W. Rhoades, B. Bartel, and D. P. Bartel.** 2002. MicroRNAs in plants. Genes Dev. **16:**1616–1626.
182. **Rhoades, M. W., B. J. Reinhart, L. P. Lim, C. B. Burge, B. Bartel, and D. P. Bartel.** 2002. Prediction of plant micro-RNA targets. Cell **110:**513–520.
183. **Robinson, K. A., and S. M. Beverley.** 2003. Improvements in transfection efficiency and tests of RNA interference (RNAi) approaches in the protozoan parasite *Leishmania*. Mol. Biochem. Parasitol. **128:**217–228.
184. **Rubinson, D. A., C. P. Dillon, A. V. Kwiatkowski, C. Sievers, L. Yang, J. Kopinja, M. Zhang, M. T. McManus, F. B. Gertler, M. L. Scott, and L. V. Parijs.** 2003. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat. Genet. **33:**401–406.
185. **Ruiz, M. T., O. Voinnet, and D. C. Baulcombe.** 1998. Initiation and maintenance of virus-induced gene silencing. Plant Cell **10:**937–946.
186. **Ruvkun, G.** 2001. Molecular biology. Glimpses of a tiny RNA world. Science **294:**797–799.
187. **Schiebel, W., T. Pelissier, L. Riedel, S. Thalmier, R. Schiebel, D. Kempe, F. Lottspeich, H. L. Sanger, and M. Wassenegger.** 1998. Isolation of an RNA-directed RNA polymerase-specific cDNA clone from tomato. Plant Cell **10:**2087–2101.
188. **Schmidt, A., G. Palumbo, M. P. Bozzetti, P. Tritto, S. Pimpinelli, and V. Schafer.** 1999. Genetic and molecular characterization of sting, a gene involved in crystal formation and meiotic drive in the male germ line of *Drosophila melanogaster*. Genetics **151:**749–760.
189. **Schwartz, D. S., G. Hutvagner, B. Haley, and P. D. Zamore.** 2002. Evidence that siRNAs function as guides, not primers, in the *Drosophila* and human pathways. Mol. Cell **10:**537–548.
190. **Semizarov, D., L. Frost, A. Sarthy, P. Kroeger, D. N. Halbert, and S. W. Fesik.** 2003. Specificity of short interfering RNA determined through gene expression signatures. Proc. Natl. Acad. Sci. USA **100:**6347–6352.
191. **Sharp, P. A.** 2001. RNA interference. Genes Dev. **15:**485–490.
192. **Shiagawa, T., and S. Ishii.** 2003. Generation of Ski-knockdown mice by expressing a long double-strand RNA from an RNA polymerase II promoter. Genes Dev. **17:**1340–1345.
193. **Sijen, T., J. Fleenor, F. Simmer, K. L. Thijssen, S. Parrish, L. Timmons, R. H. Plasterk, and A. Fire.** 2001. On the role of RNA amplification in dsRNA-triggered gene silencing. Cell **107:**465–476.
194. **Sijen, T., and J. M. Kotel.** 2000. Post-transcriptional gene silencing: RNAs on the attack or the defence. Bioassays **22:**520–531.
195. **Simmer, F., M. Tijsterman, S. Parrish, S. P. Koushika, M. L. Nonet, A. Fire, J. Ahringer, and R. H. Plasterk.** 2002. Loss of the putative RNA-directed RNA polymerase RRF-3 makes C. elegans hypersensitive to RNAi. Curr. Biol. **12:**1317–1319.
196. **Skipper, M.** 2002. RNA interference: Interfering with infection. Nat. Rev. Genet. **3:**572–573.
197. **Smardon, A., J. M. Spoerke, S. C. Stacey, M. E. Klein, N. Mackin, and, E. M. Maine.** 2000. EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in *C. elegans*. Curr. Biol. **10:**169–178.
198. **Smith, N. A., S. P. Singh, M. B. Wang, P. A. Stoutjesdijk, A. G. Green, and P. M. Waterhouse.** 2000. Total silencing by intron spliced hairpin RNA. Nature **407:**319–320.
199. **Song, E., S. K. Lee, J. Wang, N. Ince, N. Ouyang, J. Min, J. Chen, P. Shankar, and J. Libberman.** 2003. RNA interference targeting Fas protects mice from fulminant hepatitis. Nat. Med. **9:**347–351.
200. **Stauber, M., H. Taubert, and U. Schmidt-Ott.** 2000. Function of bicoid and hunchback homologs in the basal cyclorrhaphan fly *Megaselia* (Phoridae). Proc. Natl. Acad. Sci. USA **97:**10844–10849.
201. **Sullenger, B. A., and E. Gilboa.** 2002. Emerging clinical applications of RNA. Nature **418:**252–258.
202. **Tabara, H., E. Yigit, H. Siomi, and C. C. Mello.** 2002. The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExH-box helicase to direct RNAi in C. elegans. Cell **109:**861–871.
203. **Tabara, H., M. Sarkissian, W. G. Kelly, J. Fleenor, A. Grishok, L. Timmons, A. Fire, and C. C. Mello.** 1999. The rde-1 gene, RNA interference, and transposon silencing in *C. elegans*. Cell **99:**123–132.
204. **Tamaru, H., and E. U. Selker.** 2001. A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa*. Nature **414:**277–283.
205. **Tang, G., B. J. Reinhart, D. P. Bartel, and P. D. Zamore.** 2003. A biochemical framework for RNA silencing in plants. Genes Dev. **17:**49–63.
206. **Taverna, S. D., R. S. Coyne, and C. D. Allis.** 2002. Methylation of histone h3 at lysine 9 targets programmed DNA elimination in *Tetrahymena*. Cell **110:**701–711.
207. **Tenllado, F., and J. R. Diaz-Ruiz.** 2001. Double-stranded RNA-mediated interference with plant virus infection. J. Virol. **75:**12288–12297.
208. **Tijsterman, M., R. F. Ketting, K. L. Okihara, T. Sijen, and R. H. A. Plasterk.** 2002. RNA helicase MUT-14-dependent gene silencing triggered in *C. elegans* by short antisense RNAs. Science **295:**694–697.
209. **Timmons, L., and A. Fire.** 1998. Specific interference by ingested dsRNA. Nature **395:**854.
210. **Tuschl, T.** 2001. RNA interference and small interfering RNA. Chembiochem **2:**239–245.

211. **Tuschl, T., and A. Borkhardt.** 2002. Small interfering RNAs. Mol. Interv. **2**:158–167.
212. **Tuschl, T., P. D. Zamore, R. Lehmann, D. P. Bartel, and P. A. Sharp.** 1999. Targeted mRNA degradation by double-stranded RNA *in vitro*. Genes Dev. **13**:3191–3197.
213. **Ullu, E., and C. Tschudi.** 2000. RNAi: applications in parasitology. New Technol. Life Sci. **6**:43–46.
214. **Vaistij, F. E., L. Jones, and D. C. Baulcombe.** 2002. Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. Plant Cell **14**:857–867.
215. **Valdes, V. J., A. Sampieri, J. Sepulveda, and L. Vaca.** 2003. With double-stranded RNA to prevent in vitro and in vivo viral infections by recombinant baculovirus. J. Biol. Chem. **278**:19317–19324.
216. **Van Blokland, R., N. vander Geest, J. N. M. Mol, and J. M. Kooter.** 1994. Transgene-mediated suppression of chalcone synthase expression in *Petunia hybrida* results from an increase in RNA turnover. Plant J. **6**:861–877.
217. **Vander Krol, A. R., L. A. Mur, M. Beld, J. N. M. Mol, and A. R. Stuitje.** 1990. Flavanoid genes in petunia: addition of limited number of gene copies may lead to a suppression of gene expression. Plant Cell **2**:291–299.
218. **Vaucheret, H., and M. Fagard.** 2001. Transcriptional gene silencing in plants: targets, inducers and regulators. Trends Genet. **17**:29–35.
219. **Voinnet, O.** 2001. RNA silencing as a plant immune system against viruses. Trends Genet. **17**:449–459.
220. **Voinnet, O., C. Lederer, and D. C. Baulcombe.** 2000. A viral movement protein prevents spread of gene silencing signal in *Nicotiana benthamiana*. Cell **103**:157–167.
221. **Voinnet, O., P. Vain, S. Angell, and D. C. Baulcombe.** 1998. Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. Cell **95**:177–187.
222. **Voinnet, O., Y. M. Pinto, and D. C. Baulcombe.** 1999. Suppressor of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. Proc. Natl. Acad. Sci. USA **96**:14147–14152.
223. **Volpe, T. A., C. Kidner, I. M. Hall, G. Teng, S. L. S. Grewal, and R. A. Martienssen.** 2003. Regulation of heterochromatic silencing and histone H3 Lysine-9 methylation by RNAi. Science **297**:1833–1837.
224. **Wargelius, A., S. Ellingsen, and A. Fjose.** 1999. Double-stranded RNA induces specific developmental defects in zebrafish embryos. Biochem. Biophys. Res. Commun. **263**:156–161.
225. **Wassenegger, M.** 2000. RNA-directed DNA methylation. Plant Mol. Biol. **43**:203–220.
226. **Wassenegger, M., and T. Pelissier.** 1998. A model for RNA-mediated gene silencing in higher plants. Plant Mol. Biol. **37**:349–362.
227. **Waterhouse, P. M., M. B. Wang, and E. J. Finnegan.** 2001. Virus resistance and gene silencing: killing the messenger. Trends Plant Sci. **6**:297–301.
228. **Waterhouse, P. M., M. W. Graham, and M. B. Wang.** 1998. Virus resistance in gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc. Natl. Acad. Sci. USA **95**:13959–13964.
229. **Wianny, F., and M. Zernicka-Goetz.** 2000. Specific interference with gene function by double-stranded RNA in early mouse development. Nat. Cell Biol. **2**:70–75.
230. **Wilda, M., U. Fuchs, W. Wossmann, and A. Borkhardt.** 2002. Killing of leukamic cells with BCR/ABL fusion gene by RNA interference (RNAi). Oncogene. **21**:5716–5724.
231. **Winston, W. M., C. Molodowitch, and C. P. Hunter.** 2002. Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. Science **295**:2456–2459.
232. **Wu-Scharf, D., B. Jeong, C. Zhang, and H. Cerutti.** 2000. Transgene and transposon silencing in *Chlamydomonas reinhardtii* by a DEAH-box RNA helicase. Science **290**:1159–1162.
233. **Xie, Z., K. D. Kasschau, and J. C. Carrington.** 2003. Negative feedback regulation of Dicer-like1 in Arabidopsis by micro-RNA-guided mRNA degradation. Curr. Biol. **13**:784–789.
234. **Xu, P., S. Y. Vernooy, M. Guo, and B. A. Hay.** 2003. The Drosophila micro-RNA Mir-14 suppresses cell death and is required for normal fat metabolism. Curr. Biol. **13**:790–795.
235. **Yang, D., F. Buchholz, Z. Huang, A. Goga, C.-Y. Chen, F. M. Brodsky, and J. M. Bishop.** 2002. Short RNA duplexes produced by hydrolysis with RNase III mediate effective RNA interference in mammalian cells. Proc. Natl. Acad. Sci. USA **99**:9942–9947.
236. **Yang, D., H. Lu, and J. W. Erichson.** 2000. Evidence that processed small dsRNA may mediate sequence specific mRNA degradation during RNAi in *Drosophila* embryos. Curr. Biol. **10**:1191–1200.
237. **Yao, M. C., P. Fuller, and X. Xi.** 2003. Programmed DNA deletion as an RNA-guided system of genome defense. Science **300**:1581–1584.
238. **Yu, C. E., J. Oshima, Y. H. Fu, E. M. Wijsman, F. Hisama, R. Alisch, S. Matthews, J. Nakura, T. Miki, S. Ouais, G. M. Martin, J. Mulligan, and G. D. Schellenberg.** 1996. Positional cloning of the Werners syndrome gene. Science **272**:258–262.
239. **Yu, J. Y., S. L. DeRuiter, and D. L. Turner.** 2002. RNA interference by expression of short interfering RNAs and hairpin RNA by mammalian cells. Proc. Natl. Acad. Sci. USA **99**:6047–6052.
240. **Zamore, P. D., T. Tuschl, P. A. Sharp, and D. P. Bartel.** 2000. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21- to 23-nucleotide intervals. Cell **101**:25–33.
241. **Zilberman, D., X. Cao, and S. E. Jacobsen.** 2003. ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. Science **299**:716–719.

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■ REVIEW ■

RNA Interference in Functional Genomics and Medicine

RNA interference (RNAi) is the sequence-specific gene silencing induced by double-stranded RNA (dsRNA). Being a highly specific and efficient knockdown technique, RNAi not only provides a powerful tool for functional genomics but also holds a promise for gene therapy. The key player in RNAi is small RNA (~22-nt) termed siRNA. Small RNAs are involved not only in RNAi but also in basic cellular processes, such as developmental control and heterochromatin formation. The interesting biology as well as the remarkable technical value has been drawing widespread attention to this exciting new field.

Key Words : *RNA Interference (RNAi); RNA, Small interfering (siRNA); MicroRNAs (miRNA); Small hairpin RNA (shRNA); mRNA degradation; Translation; Functional genomics; Gene therapy*

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INTRODUCTION

The RNA interference (RNAi) pathway was originally recognized in *Caenorhabditis elegans* as a response to double-stranded RNA (dsRNA) leading to sequence-specific gene silencing (1). It soon turned out that RNAi is not restricted to nematode and can be induced in Drosophila (2), Trypanosoma (3), and vertebrates (4). Similar phenomena had been observed in plants and fungi, where introduction of exogenous transgenes silenced expression of the endogenous loci (5). These phenomena were called co-suppression (also termed post-transcriptional gene silencing, PTGS) and quelling, respectively. Co-suppression was shown later to be mediated by dsRNA as a guide molecule, establishing a mechanistic link to RNAi. This wide range of silencing pathways including RNAi, co-suppression (PTGS), and quelling is now collectively called RNA silencing and appears to be present in most, if not all, eukaryotic organisms. The common key player in RNA silencing is small RNA of 21-28 nucleotides (nt) in length. Two classes of small RNAs are involved in RNA silencing: small interfering RNAs (siRNAs) and microRNAs (miRNAs).

Because of the exquisite specificity and efficiency, RNAi has drawn much attention as a powerful gene knockdown technique. Previous knockdown techniques, such as antisense oligonucleotides and ribozymes, usually show low efficiency in vivo and require empirical screening of a number of candidates before acquiring effective molecules (6). SiRNAs used for RNAi can inhibit gene expression over 90% in most genes. Although a guideline for constructing the best siRNA is not established yet, testing 3-4 candidates are usually sufficient to find effective molecules. Technical expertise accumulated in the field of antisense oligonucleotide and ribozyme is now being quickly applied to RNAi, rapidly improving RNAi techniques.

In this review, the basic mechanism of small RNA-mediated silencing will be discussed. I will also describe current RNAi techniques and overview the current applications of RNAi in functional genomics and gene therapy.

SiRNA AND RNAi

RNAi is mediated by small interfering RNAs (siRNAs) that are generated from long dsRNAs of exogenous or endogenous origin (7-10). Long dsRNAs are cleaved by a ribonuclease III (RNase III) type protein Dicer. Dicer homologues can be found in *S. pombe, C. elegans,* Drosophila, plants, and mammals, suggesting that small RNA-mediated regulation is evolutionarily ancient and may have critical biological roles. SiRNA generated by Dicer is a short (~22-nt) RNA duplex with 2-nt overhang at each 3′ end (Fig. 1). Each strand contains a 5′ phosphate group and a 3′ hydroxyl group. SiRNA is incorporated into a nuclease complex called RISC (RNA-induced silencing complex) that targets and cleaves mRNA that is complementary to the siRNA. The initial RISC containing a siRNA duplex is still inactive until it is transformed into an active form (RISC*) (11), which involves loss of one strand of the duplex by an RNA helicase activity. The iden-

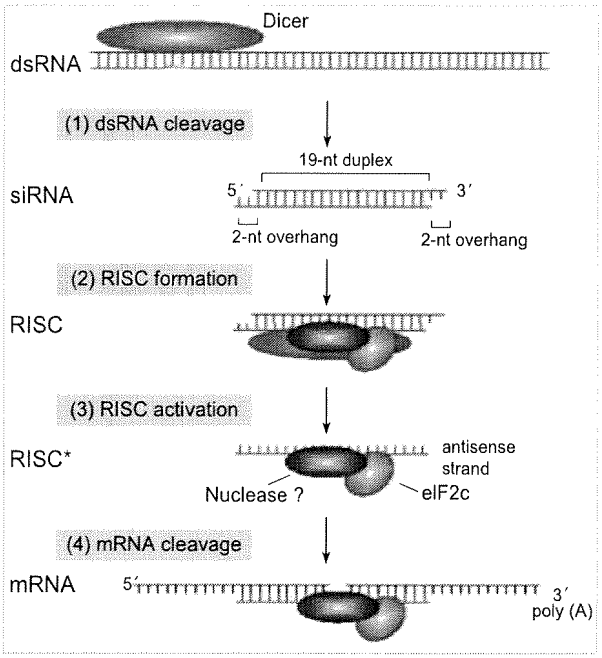

Fig. 1. Current model for RNA interference. RNAi process can be divided into four stages: (1) dsRNA cleavage by Dicer and generation of siRNA duplex, (2) recruitment of RNAi factors and formation of RISC (RNA-induced silencing complex), (3) siRNA unwinding and RISC activation, and (4) mRNA targeting and degradation.

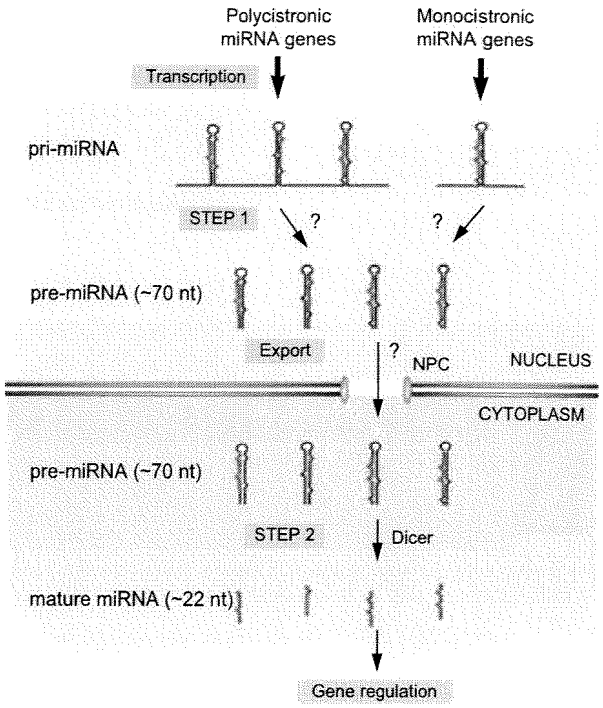

Fig. 2. A model for miRNA biogenesis and function. miRNA genes are transcribed by an unidentified polymerase to generate the primary transcripts, referred to as pri-miRNAs. Illustrated in the upper left is the clustered miRNA such as miR-23~27~24-2 of which the pri-miRNA is polycistronic. Illustrated in the upper right is the miRNA such as miR-30a of which the pri-miRNA is monocistronic. The first-step processing (STEP 1) releases pre-miRNAs of ~70-nt that is recognized and exported to the cytoplasm. The processing enzyme for the STEP 1 and the export factor are unidentified. Upon export, Dicer and possibly other factors participate in the second-step processing (STEP 2) to produce mature miRNAs. The final product may function in a variety of regulatory pathways, such as translational control of certain mRNAs. The question marks indicate unidentified factors.

tity of the RNA helicase is currently unknown. Dicer has a conserved helicase domain but it remains to be seen whether Dicer actually catalyzes this reaction.

Biochemical studies using Drosophila S2 cell extracts and human HeLa cell extracts revealed the presence of argonaute family proteins in the RISC. Argonaute-2 (AGO2) was found in Drosophila and two isoforms of eIF2C (eIF2C1 and eIF2C2) in human. Argonaute family proteins are ~100-kDa highly basic proteins that contain two common domains, PAZ and PIWI domains (12). PAZ domain consisting of ~130 amino acids is usually located at the center of the protein. The C-terminal PIWI domain containing ~300 amino acids is highly conserved. The functions of these domains are largely unknown but the PIWI domain of human eIF2C was recently shown to be essential for its interaction with Dicer (13). Depletion experiment of the eIF2C proteins by RNAi showed that they are required for RNAi (13). The biochemical functions of argonaute family proteins are still unclear.

The identity of the nuclease that executes the cleavage of mRNA remains elusive. Partially purified human RISC is estimated to be between 90 and 160-kDa leaving little room for an additional protein except for eIF2C (14). Genetic studies of *C. elegans*, Drosophila, *Neurospora crassa* and plants revealed several other genes that may be involved in RNA silencing although their biochemical roles remain to be determined.

Persistent RNAi has been observed in *C. elegans* (15) and *N. crassa* (16) but not in *D. melanogaster* (17) and mammals (18). RNAi in *C. elegans* can be transmitted to the progeny (F1) although the effect gradually diminishes. RNAi in human cells is transient and ususally lasts less than five doubling times. It was reported that SiRNAs are amplified by RNA-dependent RNA polymerase in nematode and fungi, while flies and mammals seem to lack this enzyme.

MicroRNA AND GENE SILENCING

Hundreds of small RNAs have been recently found in human as well as in *C. elegans*, *D. melanogaster*, and plants (19-29). These RNAs, termed microRNAs (miRNAs), are indistinguishable from active siRNAs in their biochemical properties. They are ~22-nt in length and contain 5′ phosphate and

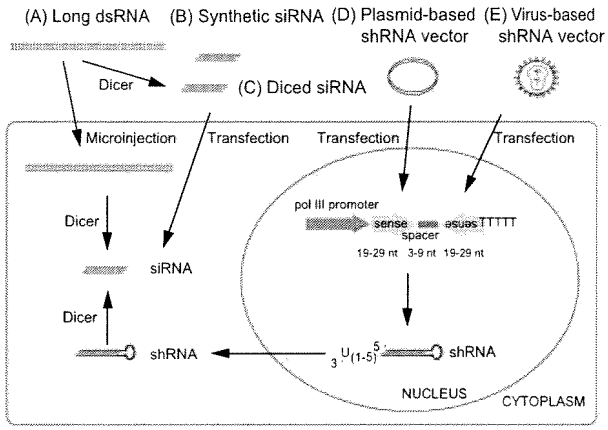

Fig. 3. Various strategies of RNAi in mammalian cells. (A) Long dsRNAs can induce specific RNAi in certain cell types such as oocytes and embryos. Injected dsRNA is converted to siRNA in the cell. (B) Chemically synthesized siRNA can be efficiently transfected into a variety of cells using lipophilic reagents. (C) SiRNA can be prepared in vitro from dsRNAs using recombinant Dicer protein. Diced RNAs are purified based on their size (~21-nt) and transfected into cells. (D) Short hairpin RNAs (shRNAs) are expressed in the nucleus from expression plasmids. The pol III-derived expression system is shown here as an example. Upon export, shRNAs are processed by Dicer releasing siRNAs. (E) ShRNA expression cassette can be delivered by viral vectors such as retroviral vector, lentiviral vector, and adenoviral vector.

3′ hydroxyl group. An interesting common feature of miRNAs, which is not shared by siRNAs, is that miRNA sequences are found in the stem of a stem-loop structure (19-22) (Fig. 2). The stem-loops are usually imperfect hairpins of over 70-nt with some bulges and internal loops. Recent studies showed that miRNAs are transcribed as long primary transcripts (pri-miRNAs) that are trimmed into the characteristic ~70-nt stem-loop forms (pre-miRNAs) (30) (Fig. 2). This initial processing occurs mainly in the nucleus (30). The identities of RNA polymerase and the nuclear processing enzyme have not been determined yet. Pre-miRNAs of ~70nt stem-loop get exported to the cytoplasm and subjected to the second processing to generate the final product of ~22-nt mature miRNAs. Dicer, the siRNA processing enzyme, executes the processing of ~70-nt RNAs into mature miRNAs (31-34).

The paradigm for the function of miRNAs has been originally provided by *lin-4* and *let-7* RNA, which were identified by genetic analysis of *C. elegans* developmental timing (35, 36). They were initially called small temporal RNAs (stRNAs) because of their temporal expression pattern and their roles in temporal regulation. *lin-4* and *let-7* RNA act as post-transcriptional repressors of their target genes when bound to their specific sites in the 3′ untranslated region of the target mRNA (35, 37-40). The level of target mRNA does not change, suggesting that the inhibition occurs at the level of translation. Recently, *bantem* RNA from Drosophila has been found to suppress apoptosis and stimulate cell proliferation by inhibiting translation of hid mRNA (41). *bantem* RNA is expressed in a temporal and tissue-specific manner, regulating tissue formation during development. Functions of hundreds of other animal miRNAs are currently unknown. However, given the diversity in sequences and expression patterns, miRNAs are expected to play various roles in a wide range of regulatory pathways (42).

Since animal miRNAs are only partially complementary to their target mRNAs (43), it is difficult to search for the target mRNA. Plant miRNAs are different from animal miRNAs in their action mechanism. Some plant miRNAs show high degree of complementarities to developmentally important mRNAs (44). Plant MIR39 and MIR165/166 were found to interact with specific mRNAs resulting in cleavage of the mRNAs, indicating that these miRNAs act like siRNAs (45, 46). Thus siRNA-mediated RNAi and miRNA-mediated translational inhibition appears to be determined only by the degree of complementarity between the small RNA and the target mRNA. Intriguingly, human *let-7* RNA was found to be a component of a RISC and can catalyze target cleavage if the mRNA has perfect complementarity to *let-7* RNA (47). Moreover, some components of miRNA-protein complex (miRNP) such as eIF2C2 overlap with those of RISC (14, 22, 47). It is an open question how RISC (RNAi machinery) and miRNP (miRNA-protein complex) are different in their compositions and functions.

PRACTICAL ASPECTS: TRANSFECTION OF SYNTHETIC siRNAS

While the discovery of RNAi revolutionized genetic studies in *C. elegans*, development of RNAi techniques in mammalian cells was belated because long dsRNA nonspecifically suppressed gene expression in mammalian cells. Long dsRNA (over 50 bp) activates dsRNA-dependent protein kinase (PKR) and 2′, 5′-oligoA synthetase leading to nonspecific translational inhibition and RNA degradation, respectively (48, 49). This pathway does not exist in embryonic stage, allowing specific RNAi in mouse oocytes and embryos (4, 50). RNAi in somatic cells was once thought to be not feasible but the limitation was soon circumvented by Thomas Tuschl's group and others by using synthetic siRNA duplexes (21-nt) that are too short to induce non-specific inhibition (51-53) (Fig. 3). This method involves transfection of synthetic siRNA into cultured cells. A detailed user guide for siRNA is given at Tuschl lab's homepage (www.rockefeller.edu/labheads/tuschl/sirna.html). Because of its straightforward protocol, siRNA transfection is the most widely used RNAi technique at present. Custom synthesis service for siRNA is available from Dharmacon RNA technologies, QIAGEN and Ambion. SiRNA can be delivered to living organisms as well, which opens the possibility of applying RNAi in gene therapy. When siRNA was inject-

ed to the tail vein of postnatal mice, it was delivered to a variety of organs including the liver, kidney, spleen, lung, and pancreas (54, 55).

There are several factors that may influence the efficiency of RNAi in mammalian system (56). First, the choice of the target site is important. Originally, it was suggested that the best target site is around the first 100-nt downstream of the translation start site. However it is not clear yet which region on mRNA is most vulnerable to RNAi. Secondary structures and mRNA-binding proteins may influence the accessibility to siRNA, although no systematic study has been carried out to compare the entire region of a given gene. In practical terms, about four different siRNAs should be randomly chosen throughout the entire mRNA and experimentally tested to find the best working one. When designing siRNA, BLAST sequence analysis is needed to avoid a chance that the designed siRNA is complementary to other unrelated mRNA. Programs to design siRNA are now available at the Whitehead Institute's biocomputing home (jura.wi.mit.edu/bio) and the siRNA manufacturers' sites (www.dharmacon.com and www.qiagen.com).

Second, the transfection method makes a difference in the outcome. SiRNAs can be efficiently transfected into cells when lipophilic agents such as Oligofectamine™ and TransIt-TKO™ are used. Oligofectamine™ is most frequently used because of its low toxicity. Virtually 100% of HeLa and HEK293 cells can be transfected using this method. Primary cells and T cell lines that are usually difficult to transfect have also been transfected with siRNAs at relatively high efficiencies. Transfection conditions such as transfection reagent, cell density, and duration of incubation should be optimized for the given cell type and the targeted gene. Apart from transfection using lipophilic agents, electroporation have been successfully used for some cell types such as T cells (57) and human hepatoma cell line, Huh-7 (58). Massive cell death during electroporation (over 50%) should be taken into consideration depending on the particular application.

Third, the turnover rate of the protein should be taken into account because RNAi only aims at the mRNA not the protein itself. Generally, siRNA causes rapid reduction in mRNA levels in 18 hr or less but stable proteins require a longer period for depletion. SiRNA-mediated RNAi lasts only for 3-5 cell doubling times, probably due to gradual dilution of siRNA through cell division. Therefore multiple transfection is necessary in cases that the protein is unusually stable or the cells need to be grown for long time to observe the phenotype.

Fourth, it is possible that two or more highly expressed genes are knocked down simultaneously but two siRNAs seem to compete with each other, suggesting that the RNAi machinery (protein factors such as argonaute family proteins) may be limiting in human cells. Thus careful control of siRNA concentration is required for simultaneous knockdown of multiple genes.

Recently, an alternative method for siRNA preparation has been developed. Long dsRNAs that are transcribed in vitro using T7 RNA polymerase (or other RNA polymerases) are incubated with recombinant Dicer to generate siRNAs (59, 60). The resulting diced products contain a mixture of siRNAs binding to multiple sites on a target mRNA, eliminating the need to design and test multiple individual siRNAs. Although this method requires longer hand-on time than synthetic siRNA method does, it is easer to design and less costly. SiRNA generation kits are available from several companies including Gene Therapy Systems, Inc.

PRACTICAL ASPECTS: DNA-BASED EXPRESSION OF SMALL HAIRPIN RNA (shRNA)

Despite of the potent knockdown capabilities, the siRNA transfection method has its weak points such as transient effect and difficulties in transfection depending on cell types. Stable gene silencing was achieved by developing a new method based on the expression of siRNAs from DNA templates (Fig. 3). The first type makes use of RNA polymerase III (pol III) promoter such as U6 promoter (61-66), H1 promoter (67-71) and $tRNA^{Val}$ promoter (72). An advantage of using the pol III system is that transcription terminates at a stretch of 4 thymidines, making it possible to produce short RNA with 1-4 uridines at the 3′ end. When "short hairpin RNA"(shRNA) similar to miRNA is transcribed from pol III promoter, shRNA gets processed by Dicer to generate siRNAs. To construct an shRNA expression cassette, the gene-specific targeting sequence (19~29-nt sequences from the target transcript separated by a short spacer from the reverse complement sequences) is inserted between pol III promoter and terminator. The loop (spacer) sequences appear to affect silencing effect. For instance, it was suggested that two uridines placed at the base of the loop were important for efficient silencing (67). Kawasaki and Taira reported that the loop sequences from miRNA (miR-23) helped the nuclear export and processing of short hairpin, enhancing RNAi effect (72). Some argue that longer stem (up to 29nt) is more efficient than shorter ones (61, 73). However, there is no clear guideline to make the best hairpin at the moment. This is partly because we do not have sufficient understanding of small RNA processing. Therefore, studies on small RNA processing would be important for development of RNAi technique.

A similar approach to the shRNA method is to transcribe ~21-nt sense and antisense RNA separately from pol III promoters (64-66). Although this method appears to provide equally strong RNAi effects, it requires construction of two expression cassettes so it does not seem to be as practical as the shRNA strategy in most cases.

ShRNAs can also be generated from pol II promoters such as human cytomegalovirus (CMV) immediate early promoter (74). Using pol II promoter would be advantageous in terms of regulated expression of siRNA. A variety of inducible/

repressible promoters are available for specific expression. This type of shRNA expression system has not been widely used yet and needs further experiments to prove its efficiency.

The first-generation RNAi vectors were plasmid-based. A selectable marker was embedded in the same plasmid, allowing selection of transfected cells. However, plasmid-based shRNA expression has limitations in cases where transfection efficiency is low.

To overcome this problem, viral vectors were employed to deliver shRNA expression cassette. Retroviral vectors are most widely used among viral vectors for in vitro gene transfer and in vivo gene therapy. Murine retrovirus-based vectors were shown to be efficient in delivery of shRNA (68, 75-78). Lentivirus-based vectors were also tested and appear to be promising vehicles for RNAi because they are effective in infecting non-cycling cells, stem cells, zygotes and their differentiated progeny (70, 79). Adenoviral vectors are highly effective but allow only transient expression of siRNA (80, 81). This property may be advantageous in some applications such as cancer gene therapy, where persisted expression is not necessary (81).

FUNCTIONAL GENOMICS

In classical forward genetics, genes were first defined by the description of their phenotype, which is then followed by the search for each gene at the molecular level. With the whole human genome sequenced and the predicted ~30,000-40,000 protein-coding genes, reverse genetics to probe the function of the predicted genes seems more effective and reasonable strategy. However, reverse genetics using knockout technique by homologous recombination takes too much time and resources. Knockdown techniques such as antisense and ribozyme proved to be far less efficient in inhibiting gene expression for this purpose.

A breakthrough was made by discovery of RNAi in *C. elegans* in 1998 when Fire et al. reported that dsRNA can induce strong and specific silencing of homologous genes (1). RNAi can be induced in this nematode worm by injection of dsRNA into gonad, by soaking the worm in dsRNA or by simply feeding the worm of bacteria engineered to express dsRNA. RNAi is now being used for studies of individual genes as well as for genome-wide genetic screening. A bacterial library for inactivation of 16,757 of the worm's predicted 19,757 genes was developed and the corresponding phenotypes were listed (82, 114). The bacterial clones are reusable and have been used in screenings for genes with more specific functions such as body fat regulation, longevity, and genome stability (83-85).

Drosophila is another popular model organism where RNAi has been successfully used to study functions of individual genes (2, 86, 87). Unlike in *C. elegans* and plants, RNAi in Drosophila is not systematic, meaning that RNAi does not spread into other cells or tissues (17, 88). This property allows cell-specific RNAi in Drosophila.

RNAi in cultured mammalian cells is quickly becoming a standard laboratory technique to study functions of individual genes. Transfection of synthetic siRNA has been most frequently used but other methods are quickly being developed as discussed above. Screening of human genome in a wider scale is also being considered. This would be much more painstaking than screening in *C. elegans* because number of human genes is about twice and RNAi technique in human is more complicated. However, efforts are already underway by developing libraries of siRNAs and automatic screening systems. Once the screening system is established, the long-standing goal of genome-wide functional genomics in human may become feasible, although the screening based on a cell line rather than the whole organism will have limitations.

RNAi may also be used to rapidly create transgenic mice. It was shown recently that retroviral or lentiviral delivery of shRNA-expression cassette can be passed through the mouse germline (79, 89). Thus, RNAi may complement standard knockout approaches and accelerate studies of gene function in living mammals.

MEDICAL APPLICATIONS

The idea of using RNAi for therapeutic purpose has been tested extensively for last two years (90) since Tuschl's pioneering work on siRNAs. Candidate diseases for such treatment include viral infection (91), cancer (92), and dominantly inherited genetic disorders.

Human immunodeficiency virus (HIV) was the first obvious target for such application. Viral genes including *tat, rev, nef,* and *gag* have been silenced, resulting in successful inhibition of viral replication in cultured cells (64, 93-99). Cellular genes such as CD4, CCR5 and CXCR4 that are necessary for viral infection were also targeted giving similar results (99, 100). Hepatitis C virus (HCV), a major cause of chronic liver disease, has a genome of a single-stranded RNA, making it an attractive target for RNAi. Expression of RNAs from HCV replicon was inhibited in cell culture, providing a hope for a new therapy for this virus (58, 101-104). Human papilloma virus (HPV) is believed to contribute to tumorigenesis. Silencing E6 and E7 genes of HPV type 16 by siRNA resulted in reduced cell growth and induced apoptosis in cervical carcinoma cells (105). Reduction in hepatitis B virus (HBV) RNAs and proteins has been induced by siRNA-producing vectors in cell culture (106) and in mouse liver (107). Influenza virus was also challenged with siRNA specific for nucleocapsid (NP) or a component of the RNA transcriptase (PA), which abolished the accumulation of viral mRNAs (108). These successful results are encouraging but there would be many hurdles to achieve viral eradication in vivo. Further experiments are needed in animal models as well as in clinical settings.

Exquisite sequence specificity of RNAi enables specific knockdown of mutated genes. Such possibility was first tested on an oncogene, *K-RAS* (V12), whose loss of expression led to loss of anchorage-independent growth and tumorigenicity (76). Employing a retroviral version of the H1 promoter-driven shRNA expression system (67), the authors showed strong inhibition of the expression of mutated *K-RAS* (V12) while leaving other ras isoforms unaffected. This approach was particularly encouraging because it was successful not only in tissue culture but also in an animal model (mouse). Similar studies quickly followed using various forms of siRNA. Oncogenes can be activated by chromosomal translocation fusing two parts of unrelated genes. M-BCR/ABL fusion leads to leukemic cells with such a rearrangement. Transfection of dsRNA specific for the *M-BCR/ABL* mRNA has been shown to downregulate the fusion protein in K562 cells (109). Overexpression of oncogenes is another cause of tumorigenesis. Overexpression of P-glycoprotein (*P-gp*), the MDR1 gene product, confers multidrug resistance (MDR) to cancer cells. RNAi successfully reduced P-gp expression and thereby drug resistance (110). Expression of endogenous erbB1 can be suppressed by RNAi in A431 human epidermoid carcinoma cells (111). Combined RNAi to reduce expression of *c-raf* and *bcl-2* genes may also represent a novel approach to leukemia (112). Blocking angiogenesis is another important anti-cancer strategy. Vascular endothelial growth factor (VEGF) exists as at least five isoforms that are thought to perform different functions in tumor angiogenesis. Specific knock-down is possible by using RNAi, providing a new tool to study isoform-specific VEGF function as well as to treat cancer (71).

Dominantly inherited genetic disorders are usually caused by mutations on one allele whose gene product acts transdominantly. Specific abrogation of the mutated gene would leave the unaffected allele to restore the normal cellular function. Expansion of trinucleotide (CAG) repeats encoding an increased polyglutamine tract causes at least eight human neurodegenerative disorders, including Huntington's disease and spinobulbar muscular atrophy (Kennedy's disease). Although the mechanism underlying neurodegeneration is unclear, aggregation of mutant polyglutamine proteins is related to the toxic gain-of-function phenotype. SiRNA targeting the 5′-end or 3′-end of the CAG repeat rescued the polyglutamine toxicity in cultured cells (80, 113), opening the possibility for new approaches.

Other diseases considered for RNAi-based therapy include Fas-induced fulminant hepatitis (55). Intravenous injection of siRNA targeting *Fas* reduced Fas expression in mouse hepatocytes leading to resistance to apoptosis and protection of mice from liver fibrosis.

There are several critical hurdles to be circumvented before RNAi becomes a realistic tool in clinics. First, enough amount of siRNA should be delivered into enough number of target cells, efficiently and stably. This delivery problem may be solved by chemically modifying siRNA to make it more stable, penetrable, and cost-effective. Alternatively, siRNAs can be delivered by way of viral vectors. Viral vectors such as lentiviral vectors would have unique advantages over synthetic siRNAs in terms of persistency. Developing optimal vectors will greatly accelerate siRNA-mediated gene therapy. A related issue is "targeted"administration of siRNA. This is hard to be achieved with synthetic siRNAs. For DNA-based RNAi, however, inducible/repressible promoters can be used to regulate siRNA expression in a tissue specific or developmental stage specific manner. The second problem stems from the technique's own merit; sequence specificity. Frequent mutations of target genes may allow escape from specific inhibition of disease genes, especially in viral infection. A "combination"strategy using several different siRNAs is likely to minimize the escape.

PERSPECTIVES

The recent discovery of small RNAs is fascinating. For decades we have been ignoring these tiny molecules as mere degradation products of bigger transcripts. Mutations in intergeneic regions were often dismissed during genetic screening. Genomics has focused on protein-coding genes leaving non-coding RNA genes unnoticed. Now hundreds of small RNA genes have been discovered. Understanding their biology is likely to reveal novel aspects of cellular functions. Key pressing questions include what their functions are and how these unusual RNA genes are expressed.

Technical aspects of small RNA biology are also important. RNAi is already changing our way of studying gene functions. However, not every promising technique is translated into commercial or clinical success. RNAi, too, should go through intensive elaboration and further innovation. Setting a guideline for selection of siRNA sequence would be an important step. Efficient delivery and regulated expression of siRNA are also critical issues for transgenic studies and gene therapy. In addition, developing a simple and inexpensive RNAi protocol for high throughput screening will be essential for large-scale genomics.

REFERENCES

1. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. *Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 1998; 391: 806-11.*
2. Kennerdell JR, Carthew RW. *Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. Cell 1998; 95: 1017-26.*
3. Ngo H, Tschudi C, Gull K, Ullu E. *Double-stranded RNA induces mRNA degradation in Trypanosoma brucei. Proc Natl Acad Sci USA 1998; 95: 14687-92.*
4. Wianny F, Zernicka-Goetz M. *Specific interference with gene func-*

tion by double-stranded RNA in early mouse development. Nat Cell Biol 2000; 2: 70-5.

5. van der Krol AR, Mur LA, Beld M, Mol JN, Stuitje AR. *Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell 1990; 2: 291-9.*
6. Aoki Y, Cioca DP, Oidaira H, Kamiya J, Kiyosawa K. *RNA interference may be more potent than antisense RNA in human cancer cell lines. Clin Exp Pharmacol Physiol 2003; 30: 96-102.*
7. Hamilton AJ, Baulcombe DC. *A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 1999; 286: 950-2.*
8. Zamore PD, Tuschl T, Sharp PA, Bartel DP. *RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 2000; 101: 25-33.*
9. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. *Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 2001; 409: 363-6.*
10. Elbashir SM, Lendeckel W, Tuschl T. *RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev 2001; 15: 188-200.*
11. Nykanen A, Haley B, Zamore PD. *ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell 2001; 107: 309-21.*
12. Carmell MA, Xuan Z, Zhang MQ, Hannon GJ. *The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. Genes Dev 2002; 16: 2733-42.*
13. Doi N, Zenno S, Ueda R, Ohki-Hamazaki H, Ui-Tei K, Saigo K. *Short-interfering-RNA-mediated gene silencing in mammalian cells requires Dicer and eIF2C translation initiation factors. Curr Biol 2003; 13: 41-6.*
14. Martinez J, Patkaniowska A, Urlaub H, Luhrmann R, Tuschl T. *Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell 2002; 110: 563-74.*
15. Sijen T, Fleenor J, Simmer F, Thijssen KL, Parrish S, Timmons L, Plasterk RH, Fire A. *On the role of RNA amplification in dsRNA-triggered gene silencing. Cell 2001; 107: 465-76.*
16. Makeyev EV, Bamford DH. *Cellular RNA-dependent RNA polymerase involved in posttranscriptional gene silencing has two distinct activity modes. Mol Cell 2002; 10: 1417-27.*
17. Roignant JY, Carre C, Mugat B, Szymczak D, Lepesant JA, Antoniewski C. *Absence of transitive and systemic pathways allows cell-specific and isoform-specific RNAi in Drosophila. RNA 2003; 9: 299-308.*
18. Chiu YL, Rana TM. *RNAi in human cells: basic structural and functional features of small interfering RNA. Mol Cell 2002; 10: 549-61.*
19. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. *Identification of novel genes coding for small expressed RNAs. Science 2001; 294: 853-8.*
20. Lau NC, Lim LP, Weinstein EG, Bartel DP. *An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science 2001; 294: 858-62.*
21. Lee RC, Ambros V. *An extensive class of small RNAs in Caenorhabditis elegans. Science 2001; 294: 862-4.*
22. Mourelatos Z, Dostie J, Paushkin S, Sharma A, Charroux B, Abel L, Rappsilber J, Mann M, Dreyfuss G. *miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes Dev 2002; 16: 720-8.*
23. Llave C, Kasschau KD, Rector MA, Carrington JC. *Endogenous and silencing-associated small RNAs in plants. Plant Cell 2002; 14: 1605-19.*
24. Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, Bartel DP. *MicroRNAs in plants. Genes Dev 2002; 16: 1616-26.*
25. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. *Identification of tissue-specific microRNAs from mouse. Curr Biol 2002; 12: 735-9.*
26. Park W, Li J, Song R, Messing J, Chen X. *CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in Arabidopsis thaliana. Curr Biol 2002; 12: 1484-95.*
27. Lagos-Quintana M, Rauhut R, Meyer J, Borkhardt A, Tuschl T. *New microRNAs from mouse and human. RNA 2003; 9: 175-9.*
28. Dostie J, Mourelatos Z, Yang M, Sharma A, Dreyfuss G. *Numerous microRNPs in neuronal cells containing novel microRNAs. RNA 2003; 9: 180-6.*
29. Lim LP, Lau NC, Weinstein EG, Abdelhakim A, Yekta S, Rhoades MW, Burge CB, Bartel DP. *The microRNAs of Caenorhabditis elegans. Genes Dev 2003; 17: 991-1008.*
30. Lee Y, Jeon K, Lee JT, Kim S, Kim VN. *MicroRNA maturation: stepwise processing and subcellular localization. EMBO J 2002; 21: 4663-70.*
31. Knight SW, Bass BL. *A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in Caenorhabditis elegans. Science 2001; 293: 2269-71.*
32. Ketting RF, Fischer SE, Bernstein E, Sijen T, Hannon GJ, Plasterk RH. *Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev 2001; 15: 2654-9.*
33. Hutvagner G, McLachlan J, Pasquinelli AE, Balint E, Tuschl T, Zamore PD. *A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science 2001; 293: 834-8.*
34. Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S, Ha I, Baillie DL, Fire A, Ruvkun G, Mello CC. *Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell 2001; 106: 23-34.*
35. Lee RC, Feinbaum RL, Ambros V. The C. *elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 1993; 75: 843-54.*
36. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G. *The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 2000; 403: 901-6.*
37. Wightman B, Ha I, Ruvkun G. *Posttranscriptional regulation of the heterochronic gene lin-14 by lin- 4 mediates temporal pattern formation in C. elegans. Cell 1993; 75: 855-62.*
38. Moss EG, Lee RC, Ambros V. *The cold shock domain protein LIN-28 controls developmental timing in C. elegans and is regulated by the lin-4 RNA. Cell 1997; 88: 637-46.*
39. Olsen PH, Ambros V. *The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. Dev Biol 1999; 216: 671-*

80.

40. Slack FJ, Basson M, Liu Z, Ambros V, Horvitz HR, Ruvkun G. *The lin-41 RBCC gene acts in the C. elegans heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. Mol Cell 2000; 5: 659-69.*
41. Brennecke J, Hipfner DR, Stark A, Russell RB, Cohen SM. *bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. Cell 2003; 113: 25-36.*
42. Lai EC. *Micro RNAs are complementary to 3′ UTR sequence motifs that mediate negative post-transcriptional regulation. Nat Genet 2002; 30: 363-4.*
43. Ruvkun G. *Molecular biology. Glimpses of a tiny RNA world. Science 2001; 294: 797-9.*
44. Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP. *Prediction of plant microRNA targets. Cell 2002; 110: 513-20.*
45. Llave C, Xie Z, Kasschau KD, Carrington JC. *Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. Science 2002; 297: 2053-6.*
46. Tang G, Reinhart BJ, Bartel DP, Zamore PD. *A biochemical framework for RNA silencing in plants. Genes Dev 2003; 17: 49-63.*
47. Hutvagner G, Zamore PD. *A microRNA in a multiple-turnover RNAi enzyme complex. Science 2002; 297: 2056-60.*
48. Gil J, Esteban M. *Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): mechanism of action. Apoptosis 2000; 5: 107-14.*
49. Kumar M, Carmichael GG. *Antisense RNA: function and fate of duplex RNA in cells of higher eukaryotes. Microbiol Mol Biol Rev 1998; 62: 1415-34.*
50. Svoboda P, Stein P, Hayashi H, Schultz RM. *Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference. Development 2000; 127: 4147-56.*
51. Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, Tuschl T. *Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. EMBO J 2001; 20: 6877-88.*
52. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. *Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001; 411: 494-8.*
53. Caplen NJ, Parrish S, Imani F, Fire A, Morgan RA. *Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. Proc Natl Acad Sci USA 2001; 98: 9742-7.*
54. Lewis DL, Hagstrom JE, Loomis AG, Wolff JA, Herweijer H. *Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. Nat Genet 2002; 32: 107-8.*
55. Song E, Lee SK, Wang J, Ince N, Ouyang N, Min J, Chen J, Shankar P, Lieberman J. *RNA interference targeting Fas protects mice from fulminant hepatitis. Nat Med 2003; 9: 347-51.*
56. McManus MT, Sharp PA. *Gene silencing in mammals by small interfering RNAs. Nat Rev Genet 2002; 3: 737-47.*
57. McManus MT, Haines BB, Dillon CP, Whitehurst CE, van Parijs L, Chen J, Sharp PA. *Small interfering RNA-mediated gene silencing in T lymphocytes. J Immunol 2002; 169: 5754-60.*
58. Wilson JA, Jayasena S, Khvorova A, Sabatinos S, Rodrigue-Gervais IG, Arya S, Sarangi F, Harris-Brandts M, Beaulieu S, Richardson CD. *RNA interference blocks gene expression and RNA synthesis from hepatitis C replicons propagated in human liver cells. Proc Natl Acad Sci USA 2003; 100: 2783-8.*
59. Myers JW, Jones JT, Meyer T, Ferrell JE. *Recombinant Dicer efficiently converts large dsRNAs into siRNAs suitable for gene silencing. Nat Biotechnol 2003; 21: 324-8.*
60. Kawasaki H, Suyama E, Iyo M, Taira K. *siRNAs generated by recombinant human Dicer induce specific and significant but target site-independent gene silencing in human cells. Nucleic Acids Res 2003; 31: 981-7.*
61. Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, Conklin DS. *Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev 2002; 16: 948-58.*
62. Sui G, Soohoo C, Affar el B, Gay F, Shi Y, Forrester WC. *A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc Natl Acad Sci USA 2002; 99: 5515-20.*
63. Paul CP, Good PD, Winer I, Engelke DR. *Effective expression of small interfering RNA in human cells. Nat Biotechnol 2002; 20: 505-8.*
64. Lee NS, Dohjima T, Bauer G, Li H, Li MJ, Ehsani A, Salvaterra P, Rossi J. *Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. Nat Biotechnol 2002; 20: 500-5.*
65. Yu JY, DeRuiter SL, Turner DL. *RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. Proc Natl Acad Sci USA 2002; 99: 6047-52.*
66. Miyagishi M, Taira K. *U6 promoter-driven siRNAs with four uridine 3′ overhangs efficiently suppress targeted gene expression in mammalian cells. Nat Biotechnol 2002; 20: 497-500.*
67. Brummelkamp TR, Bernards R, Agami R. *A system for stable expression of short interfering RNAs in mammalian cells. Science 2002; 296: 550-3.*
68. Barton GM, Medzhitov R. *Retroviral delivery of small interfering RNA into primary cells. Proc Natl Acad Sci USA 2002; 99: 14943-5.*
69. McManus MT, Petersen CP, Haines BB, Chen J, Sharp PA. *Gene silencing using micro-RNA designed hairpins. RNA 2002; 8: 842-50.*
70. Abbas-Terki T, Blanco-Bose W, Deglon N, Pralong W, Aebischer P. *Lentiviral-mediated RNA interference. Hum Gene Ther 2002; 13: 2197-201.*
71. Zhang L, Yang N, Mohamed-Hadley A, Rubin SC, Coukos G. *Vector-based RNAi, a novel tool for isoform-specific knock-down of VEGF and anti-angiogenesis gene therapy of cancer. Biochem Biophys Res Commun 2003; 303: 1169-78.*
72. Kawasaki H, Taira K. *Short hairpin type of dsRNAs that are controlled by tRNA (Val) promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells. Nucleic Acids Res 2003; 31: 700-7.*
73. Yu JY, Taylor J, DeRuiter SL, Vojtek AB, Turner DL. *Simultaneous inhibition of GSK3alpha and GSK3beta using hairpin siRNA expression vectors. Mol Ther 2003; 7: 228-36.*
74. Zeng Y, Wagner EJ, Cullen BR. *Both natural and designed Micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. Mol Cell 2002; 9: 1327-33.*
75. Devroe E, Silver PA. *Retrovirus-delivered siRNA. BMC Biotechnol 2002; 2: 15.*
76. Brummelkamp TR, Bernards R, Agami R. *Stable suppression of tu-*

morigenicity by virus-mediated RNA interference. Cancer Cell 2002; 2: 243-7.

77. Stewart SA, Dykxhoorn DM, Palliser D, Mizuno H, Yu EY, An DS, Sabatini DM, Chen IS, Hahn WC, Sharp PA, Weinberg RA, Novina CD. *Lentivirus-delivered stable gene silencing by RNAi in primary cells. RNA 2003; 9: 493-501.*
78. Hemann MT, Fridman JS, Zilfou JT, Hernando E, Paddison PJ, Cordon-Cardo C, Hannon GJ, Lowe SW. *An epi-allelic series of p53 hypomorphs created by stable RNAi produces distinct tumor phenotypes in vivo. Nat Genet 2003; 33: 396-400.*
79. Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, Kopinja J, Zhang M, McManus MT, Gertler FB, Scott ML, Van Parijs L. *A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat Genet 2003; 33: 401-6.*
80. Xia H, Mao Q, Paulson HL, Davidson BL. *siRNA-mediated gene silencing in vitro and in vivo. Nat Biotechnol 2002; 20: 1006-10.*
81. Shen C, Buck AK, Liu X, Winkler M, Reske SN. *Gene silencing by adenovirus-delivered siRNA. FEBS Lett 2003; 539: 111-4.*
82. Kamath RS, Fraser AG, Dong Y, Poulin G, Durbin R, Gotta M, Kanapin A, Le Bot N, Moreno S, Sohrmann M, Welchman DP, Zipperlen P, Ahringer J. *Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. Nature 2003; 421: 231-7.*
83. Ashrafi K, Chang FY, Watts JL, Fraser AG, Kamath RS, Ahringer J, Ruvkun G. *Genome-wide RNAi analysis of Caenorhabditis elegans fat regulatory genes. Nature 2003; 421: 268-72.*
84. Lee SS, Lee RY, Fraser AG, Kamath RS, Ahringer J, Ruvkun G. *A systematic RNAi screen identifies a critical role for mitochondria in C. elegans longevity. Nat Genet 2003; 33: 40-8.*
85. Pothof J, Van Haaften G, Thijssen K, Kamath RS, Fraser AG, Ahringer J, Plasterk RH, Tijsterman M. *Identification of genes that protect the C. elegans genome against mutations by genome-wide RNAi. Genes Dev 2003; 17: 443-8.*
86. Schmid A, Schindelholz B, Zinn K. *Combinatorial RNAi: a method for evaluating the functions of gene families in Drosophila. Trends Neurosci 2002; 25: 71-4.*
87. Kim C, Han K, Kim J, Yi JS, Yim J, Kim YJ, Kim-Ha J. *Femcoat, a novel eggshell protein in Drosophila: functional analysis by double stranded RNA interference. Mech Dev 2002; 110: 61-70.*
88. Billuart P, Winter CG, Maresh A, Zhao X, Luo L. *Regulating axon branch stability: the role of p190 RhoGAP in repressing a retraction signaling pathway. Cell 2001; 107: 195-207.*
89. Carmell MA, Zhang L, Conklin DS, Hannon GJ, Rosenquist TA. *Germline transmission of RNAi in mice. Nat Struct Biol 2003; 10: 91-2.*
90. Shuey DJ, McCallus DE, Giordano T. *RNAi: gene-silencing in therapeutic intervention. Drug Discov Today 2002; 7: 1040-6.*
91. Silva JM, Hammond SM, Hannon GJ. *RNA interference: a promising approach to antiviral therapy? Trends Mol Med 2002; 8: 505-8.*
92. Borkhardt A. *Blocking oncogenes in malignant cells by RNA interference--new hope for a highly specific cancer treatment? Cancer Cell 2002; 2: 167-8.*
93. Capodici J, Kariko K, Weissman D. *Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference. J Immunol 2002; 169: 5196-201.*
94. Coburn GA, Cullen BR. *Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. J Virol 2002; 76: 9225-31.*
95. Hu WY, Myers CP, Kilzer JM, Pfaff SL, Bushman FD. *Inhibition of retroviral pathogenesis by RNA interference. Curr Biol 2002; 12: 1301-11.*
96. Jacque JM, Triques K, Stevenson M. *Modulation of HIV-1 replication by RNA interference. Nature 2002; 418: 435-8.*
97. Yamamoto T, Omoto S, Mizuguchi M, Mizukami H, Okuyama H, Okada N, Saksena NK, Brisibe EA, Otake K, Fuji YR. *Double-stranded nef RNA interferes with human immunodeficiency virus type 1 replication. Microbiol Immunol 2002; 46: 809-17.*
98. Park WS, Miyano-Kurosaki N, Hayafune M, Nakajima E, Matsuzaki T, Shimada F, Takaku H. *Prevention of HIV-1 infection in human peripheral blood mononuclear cells by specific RNA interference. Nucleic Acids Res 2002; 30: 4830-5.*
99. Novina CD, Murray MF, Dykxhoorn DM, Beresford PJ, Riess J, Lee SK, Collman RG, Lieberman J, Shankar P, Sharp PA. *siRNA-directed inhibition of HIV-1 infection. Nat Med 2002; 8: 681-6.*
100. Martinez MA, Gutierrez A, Armand-Ugon M, Blanco J, Parera M, Gomez J, Clotet B, Este JA. *Suppression of chemokine receptor expression by RNA interference allows for inhibition of HIV-1 replication. AIDS 2002; 16: 2385-90.*
101. Randall G, Grakoui A, Rice CM. *Clearance of replicating hepatitis C virus replicon RNAs in cell culture by small interfering RNAs. Proc Natl Acad Sci USA 2003; 100: 235-40.*
102. Kapadia SB, Brideau-Andersen A, Chisari FV. *Interference of hepatitis C virus RNA replication by short interfering RNAs. Proc Natl Acad Sci USA 2003; 100: 2014-8.*
103. Seo MY, Abrignani S, Houghton M, Han JH. *Small interfering RNA-mediated inhibition of hepatitis C virus replication in the human hepatoma cell line Huh-7. J Virol 2003; 77: 810-2.*
104. Yokota T, Sakamoto N, Enomoto N, Tanabe Y, Miyagishi M, Maekawa S, Yi L, Kurosaki M, Taira K, Watanabe M, Mizusawa H. *Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. EMBO Rep 2003; 4: 1-7.*
105. Jiang M, Milner J. *Selective silencing of viral gene expression in HPV-positive human cervical carcinoma cells treated with siRNA, a primer of RNA interference. Oncogene 2002; 21: 6041-8.*
106. Shlomai A, Shaul Y. *Inhibition of hepatitis B virus expression and replication by RNA interference. Hepatology 2003; 37: 764-70.*
107. McCaffrey AP, Nakai H, Pandey K, Huang Z, Salazar FH, Xu H, Wieland SF, Marion PL, Kay MA. *Inhibition of hepatitis B virus in mice by RNA interference. Nat Biotechnol 2003; 21: 639-44.*
108. Ge Q, McManus MT, Nguyen T, Shen CH, Sharp PA, Eisen HN, Chen J. *RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. Proc Natl Acad Sci USA 2003; 100: 2718-23.*
109. Wilda M, Fuchs U, Wossmann W, Borkhardt A. *Killing of leukemic cells with a BCR/ABL fusion gene by RNA interference (RNAi). Oncogene 2002; 21: 5716-24.*
110. Wu H, Hait WN, Yang JM. *Small interfering RNA-induced suppression of MDR1 (P-glycoprotein) restores sensitivity to multidrug-resistant cancer cells. Cancer Res 2003; 63: 1515-9.*

111. Nagy P, Arndt-Jovin DJ, Jovin TM. *Small interfering RNAs suppress the expression of endogenous and GFP-fused epidermal growth factor receptor (erbB1) and induce apoptosis in erbB1-overexpressing cells. Exp Cell Res 2003; 285: 39-49.*

112. Cioca DP, Aoki Y, Kiyosawa K. *RNA interference is a functional pathway with therapeutic potential in human myeloid leukemia cell lines. Cancer Gene Ther 2003; 10: 125-33.*

113. Caplen NJ, Taylor JP, Statham VS, Tanaka F, Fire A, Morgan RA. *Rescue of polyglutamine-mediated cytotoxicity by double-stranded RNA- mediated RNA interference. Hum Mol Genet 2002; 11: 175-84.*

114. Maeda I, Kohara Y, Yamamoto M, Sugimoto A. *Large-scale analysis of gene function in Caenorhabditis elegans by high- throughput RNAi. Curr Biol 2001; 11: 171-6.*

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MINIREVIEW

Nucleic Acid-Based Immune System: the Antiviral Potential of Mammalian RNA Silencing

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The discovery of a highly conserved cellular machinery that can regulate gene expression in response to double-stranded RNA may revolutionize mammalian virology. This revolution promises not only a deeper understanding of host-pathogen interactions and a novel set of experimental tools to explore the mechanism of viral replication but may also yield new therapeutic approaches. Even though the field of RNA silencing (or RNA interference [RNAi]) as applied to mammalian viruses is barely a year old, there is already enough material to appreciate its importance and to discuss its implications. Since an impressive number of excellent reviews on RNAi have been published recently (25, 27, 62), here we emphasize mammalian RNA silencing as it concerns one of its (presumably) natural targets, viruses.

RNAi IN PLANTS AND INVERTEBRATES

RNAi, also known as RNA silencing or posttranscriptional gene silencing, is a process that responds to double-stranded RNA (dsRNA) by silencing gene expression in a sequence-specific manner. The RNAi machinery uses dsRNA duplexes as guides to target and destroy specific cellular or viral RNAs. RNA silencing was initially described in plants more than a decade ago (reviewed in reference 62). Overexpression of transgenes in plants can result in cosuppression of homologous endogenous genes (43, 63). Also, infection by plant RNA viruses can be restricted or prevented by the artificial expression of pieces of the viral genomic RNA. This resistant state appears to be mediated by a cytoplasmic activity that targets specific RNAs for inactivation (38). It was thus proposed that suppression of endogenous genes or resistance to RNA viruses could arise from a sequence-specific RNA degradation system. Similar mechanisms were invoked to explain the phenomenon of cross-protection in which a nonpathogenic strain of plant virus elicits resistance to a related pathogenic virus (reviewed in reference 53). Furthermore, it was observed that cellular genes carried by viruses could lead to decreased expression of cognate endogenous host genes, an occurrence that was suggested to be mediated by the same mechanism responsible for virus resistance and which was named virus-induced gene silencing (33).

The critical role of dsRNA in initiating a process of sequence-specific mRNA degradation was first described for the nematode *Caenorhabditis elegans* by the artificial introduction of dsRNA (19). This process was called RNAi and has since been observed in a variety of eukaryotes (31, 44, 67). The emerging view from these studies is that the central player in the RNA silencing pathways is dsRNA, which acts as a trigger and/or intermediate of the process (Fig. 1). Thus, RNA silencing can be triggered by viruses or transposons that generate dsRNA during their replication or artificially by the introduction of synthetic dsRNA. It has also been proposed in plants that "aberrant RNAs" are capable of initiating RNA silencing responses (65). However, these aberrant RNAs have not been well characterized and are likely to contain dsRNA structures or motifs. The initial dsRNA is then cleaved into small, interfering dsRNAs (siRNAs), 21 to 25 nucleotides long, by a protein complex containing Dicer and possibly homologues of the *C. elegans* genes *rde-4*, *rde-1*, and *drh-1/2* (58). The siRNAs in turn are incorporated into the RNA-induced silencing complex (RISC), and they are unwound presumably to act as guides to direct the RNA degradation machinery to the target RNAs (41). The RISC monitors the sequence of cytoplasmic RNAs and is able to cleave the cognate target RNA in a sequence-specific, siRNA-dependent manner (41). Although not all the components of the RISC have been identified, it appears that one or more members of the Argonaute gene family, such as the eukaryotic initiation factor 2C proteins, form part of the complex (41). The RNA silencing machinery also appears to be able to amplify the degradation signal. It has been proposed for invertebrates that siRNAs can function as primers that are extended on the targeted RNA by an RNA-dependent RNA polymerase (RdRp) (39, 54). This amplification has not yet been observed in mammalian systems.

It is now generally accepted that RNA silencing is a major antiviral defense mechanism in plants (3a, 17a, 48, 50a). Although the natural trigger for RNA silencing in virally infected cells is presently unknown, because RNA viruses replicate through dsRNA intermediates it is possible that these replication intermediates serve to initiate the RNA silencing response. Even DNA viruses may produce dsRNAs as by-products of bidirectional transcription from their genome (15, 34). Several lines of evidence support the concept that RNA silencing is an antiviral mechanism in plants. First, as described

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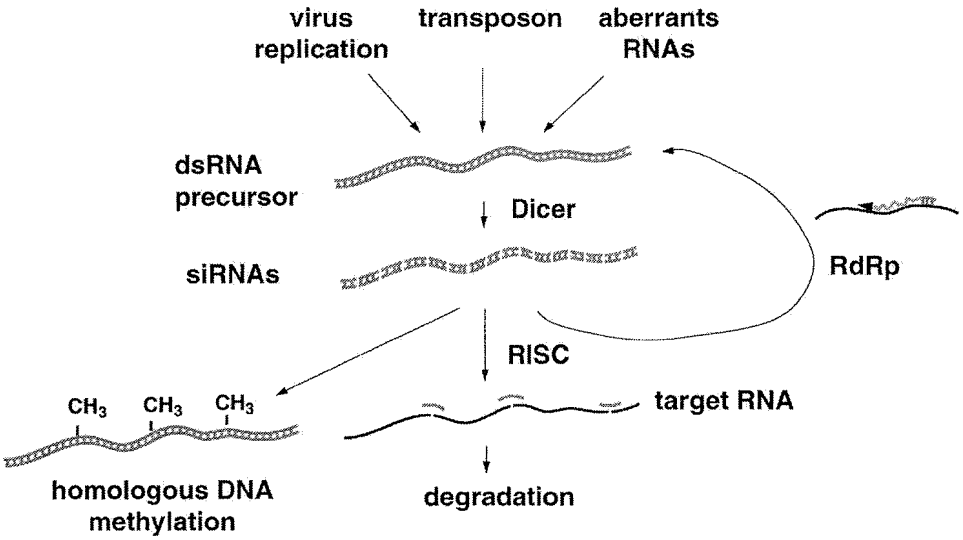

FIG. 1. Schematic representation of the molecular mechanism of the RNA silencing pathway. The process can be triggered by viral infection, transposons, or aberrant RNAs, all of which produce some form of dsRNA. Long dsRNA precursors are processed by Dicer to siRNAs. siRNAs associate with the RISC, which in turn targets and induces degradation of specific RNAs. The siRNAs can be amplified by a cellular RdRp. The RNA silencing system has also been associated with DNA methylation.

above, natural infection by plant viruses elicits strong gene silencing (38). This observation indicates that the viral genomic RNA or intermediates of replication are used effectively by silencing machinery to trigger RNA silencing. Second, viral replication can be efficiently suppressed by experimentally induced RNA silencing (38, 66). Third, plant viruses encode a variety of inhibitors of the RNA silencing machinery (5, 11, 30, 64). The fact that viruses have acquired mechanisms to interfere with RNA silencing suggests that, for successful replication in nature, viruses need to overcome the plant's antiviral defenses. Fourth, some components of the RNA silencing machinery, like the RdRp, are upregulated by viroid and virus infection (6, 51). Perhaps the most persuasive indication that RNA silencing is an important antiviral defense mechanism in plants is the observation that mutations in genes that encode the RNA silencing machinery, like *sgs2* (also called *sde1*), *sgs3*, and *sde3*, result in enhanced susceptibility to virus infection (16, 42). Taken together, these are strong indications that RNAi is an antiviral mechanism in plants.

RNA silencing also appears to contribute to antiviral defense in invertebrates. Preinfection of cells or whole mosquitoes with Sindbis virus carrying dengue virus genome fragments was shown to inhibit dengue virus replication (1, 20, 46). Although these results are reminiscent of the cross-protection phenomenon seen in plants (53), these experiments could not distinguish whether the inhibition was produced by RNA silencing, RNA antisense activity, or dominant-negative effects, such as those induced by defective interfering RNAs. However, a recent study demonstrated that it is indeed possible to inhibit dengue virus production by transfection of dsRNA in mosquito cells; in contrast, a single-stranded RNA control was not effective (12). Furthermore, cells transfected with a plasmid designed to express an inverted-repeat RNA derived from the dengue virus genome were resistant to virus infection and accumulated short species of dsRNAs (siRNAs) (2).

Important evidence supporting a physiological antiviral role for RNAi in invertebrates was obtained by studies of flock house virus (FHV) and its interaction with the RNA silencing machinery in *Drosophila* cells (37). This nodavirus infects insects but can also replicate in plant and mammalian cells. FHV infection results in accumulation of siRNAs specific for the viral genome. These siRNAs are able to promote the specific degradation of artificially introduced viral RNA. Interestingly, FHV protein B2 can block RNA silencing in both plant and invertebrate cells. B2 can functionally replace the 2b protein of cucumber mosaic virus, which belongs to a group of well-known suppressors of RNA silencing. Notably, FHV-induced RNA silencing was prevented by expression of B2 or depletion of AGO2 (a putative RISC component of the Argonaute family). These experiments argue for a role of RNA silencing as an adaptive antiviral defense in invertebrates. They also raise the question of how RNAi acts in response to natural viral infection and whether, as observed in plants, RNAi in insects can induce a systemic protection against viruses.

siRNAs AS AN ANTIVIRAL MECHANISM IN MAMMALIAN CELLS

Is there a mammalian antiviral RNAi counterpart? It was originally thought that mammalian cells were unlikely to possess an active RNA silencing machinery (19). This skepticism was derived from the fact that mammalian cells respond to the presence of dsRNA with a general, nonspecific shutdown of translation, mediated primarily by protein kinase R (PKR) and RNase L. Indeed, this response is considered an important innate antiviral mechanism that is upregulated by interferon and contributes to controlling viral infection in a nonspecific fashion (36, 55). Furthermore, mammals have evolved a sophisticated immune system based on protein recognition that protects them against infection in a highly specific manner.

This system is not present in plants and invertebrates and thus could have functionally replaced the RNA silencing system. Yet it would seem that such a versatile antiviral system as RNA silencing might be conserved during evolution, since viruses (as well as other molecular parasites, like transposons) probably maintain an unrelenting selective pressure on their hosts.

Indeed, the recent description of RNAi in mammalian cells proved that the RNA silencing machinery is conserved in mammals (9, 12, 57, 61, 67). These studies showed that while the PKR-RNase L pathway is activated by long dsRNA in most cells, siRNAs (21 nucleotides long) are readily recognized by a mammalian RISC and mediate significant reduction in the levels of a specific target mRNA. These observations led several groups to explore the interaction between the RNA silencing machinery and mammalian viruses.

The initial experiments centered on a simple question. Are mammalian viruses susceptible to RNAi? While potentially any RNA can be subject to degradation by the RNAi machinery, it was not clear whether viral RNAs could be effectively targeted. Virus genomes are often protected by a proteinaceous structure (dsRNA viruses), by nucleoproteins and matrix layers (negative-stranded RNA viruses), or by association with cellular membranes during replication (positive-stranded RNA viruses). In addition, a large part of the newly synthesized RNA is often rapidly encapsidated in new virus particles. Thus, the recent discovery that siRNAs can block replication of several different types of mammalian viruses is significant, especially given its therapeutic implications. siRNA has been shown to inhibit production of two retroviruses, the human immunodeficiency virus (HIV) (13, 14, 26, 28, 35, 45, 48, 56) and Rous sarcoma virus (26); a negative-stranded RNA virus, respiratory syncytial virus (10); and a positive-stranded RNA virus, poliovirus (21), and to inhibit the gene expression of a DNA virus, human papillomavirus (29). In all these reports, cells transfected with siRNA corresponding to the viral genome induced a clear reduction in virus production. In general, a decrease in titer of 10- to 200-fold was observed (14, 21, 26, 28, 45). All these studies demonstrate that accumulation of viral RNA in infected cells was significantly reduced by siRNAs, indicating that viral RNA can be targeted by the RNA silencing machinery.

The action of siRNAs appears to be independent of the nonspecific PKR-RNase L responses. Thus, control siRNAs of unrelated sequences failed to inhibit virus production (13, 14, 21, 26, 28, 45, 56). Also, siRNA transfection did not induce phosphorylation of PKR (10, 28), a signal of interferon-mediated activation of the innate defense system (55). Furthermore, transfection of siRNAs into mouse embryonic fibroblasts deficient in both PKR and RNase L was effective in inhibiting virus replication (21). In addition, the antiviral siRNAs did not result in interferon production, as viral resistance could not be induced by culturing cells in medium containing supernatant from siRNA-transfected cells (21). These studies suggest that the classical interferon and nonspecific dsRNA pathways are not part of the specific RNA silencing mechanism.

An important question raised by these recent studies concerns the duration and amplification of the siRNA signal and effect. The ability of siRNA-transfected cells to resist virus infection was maintained over the course of several days (21, 28). The duration of the effect could either indicate that the interference state persists for a few days or, alternatively, that the siRNAs could be slowly released from the cell-associated transfection mixture. Assuming that cells can indeed maintain the silencing state for several days, a remarkable parallel to the protein-based immune system can be drawn, whereby the RNA silencing system induces a specific response which can last for at least a few days. Perhaps the system is capable of establishing a rudimentary form of memory. It would be interesting to determine whether siRNAs are preserved freely in the cytoplasm or are associated with components of the RNAi machinery in order to resist degradation. Perhaps, transfected siRNAs are able to associate with the RISC or even with the hypothetical RdRp and in this manner not only prolong their half-lives but also amplify the signal over a certain period of time.

Another important question is whether viral nucleoproteins and/or replication strategies play a role in shielding the viral genome from the RNAi machinery (discussed in reference 3). At this stage, the question has mainly been addressed for HIV. Since different studies have used different siRNAs, cell types, viral strains, multiplicities of infection, and times between transfection and infection, the answer is not yet conclusive. However, there is a consensus that expression of HIV RNA from proviral DNA can be effectively inhibited by RNAi (26, 28, 35, 45). What is unclear at this time is whether RNAi can target the incoming viral RNA while in transit to the nucleus, when it is still associated with nucleocapsid proteins. Using PCR to detect integrated viral genomes, Hu and colleagues (26) found that the number of integrated proviruses is identical in HIV siRNA- and control siRNA-treated cells, suggesting that the incoming viral RNA is inaccessible to RNAi. In contrast, Coburn and Cullen's study, using hybridization to detect viral genomic DNA in infected cells, revealed a difference between antiviral siRNA-treated and control samples (14). Consistent with this result, Capodici et al. observed a clear reduction in the amount of HIV DNA that accumulated in cells treated with siRNAs (13) and Jacque et al. found a significant difference in HIV genomic RNA in siRNA-treated cells as early as 1 h after infection, indicating that incoming RNA can be targeted by RNAi (28). A critical difference between these studies is that they target different sequences within the HIV genome. This difference can account for the discrepant results since the nucleocapsid of the incoming virus may preferentially shield some regions of the RNA. For negative-stranded RNA viruses, it appears that Rous sarcoma virus mRNA is susceptible to siRNA targeting, but genomic RNA, which is known to be coated by a nucleoprotein, is not (10).

Since all the studies on antiviral activity of RNAi have employed defined, short RNA sequences (siRNAs or short hairpin RNAs [shRNAs]), they allow evaluation of the tolerance of the RNAi machinery to mismatches. It appears that mismatches between the siRNA and its viral target generally are not well tolerated. One mismatch located approximately in the center of the siRNA nearly abolishes silencing of poliovirus (L. Gitlin and R. Andino, unpublished data). However, certain anti-HIV siRNAs carrying a single-nucleotide mismatch can be as effective as those with sequences that perfectly match the target RNA (28). The tolerance of RNA silencing to mismatches is important because it relates to the ability of viruses to escape inhibition by RNA silencing. If the RNAi machinery

can accommodate mismatches, it would be more difficult for the virus to escape the action of siRNA. The fact that a single point mutation yielded an RNAi-resistant virus raises an important challenge for the use of short, defined siRNAs in therapeutic approaches. On the other hand, a "natural" RNA silencing response that targets multiple viral sequences would be harder to evade, as escape would require extensive alterations in the viral genome.

The viruses which are least likely to escape from siRNAs are the DNA viruses, due to their lower mutation frequency. Among them, an important therapeutic target is papillomaviruses, because it appears that the constitutive expression of viral proteins E6 and E7 is required for carcinogenic growth (18). Recent results with siRNAs targeting E6 and E7 in cultured cells suggested that E6 downregulation by siRNAs can lead to cell growth suppression, while E7 downregulation results in apoptosis (29).

A striking conclusion from one of the recent studies is that siRNAs can clear viral infection without causing any visible harm to the infected cell (21). It has been a long-standing assumption in immunology that clearance of virus from the mammalian host requires destruction of infected cells (22), either by the action of the immune system or by apoptosis induced by the virus. This idea has been challenged in studies that observed reduction and even clearance of viral genomes in the absence of significant cytopathology (23, 32). Although the mechanism that induced viral clearance in these systems is unknown, it is tempting to speculate that it could be mediated by RNA silencing. Indeed, siRNA can effectively clear poliovirus from infected HeLa cells (21). A recent study has also demonstrated that siRNAs can be effective in clearing hepatitis C virus replicons (50). These experiments highlight the potential of RNA silencing as an agent of noncytopathic viral clearance.

FUTURE DIRECTIONS

These initial studies of the effect of RNA silencing on viral replication in mammalian cells open two major avenues of investigation. First, it will be important to understand the role (if any) of RNA silencing in providing an antiviral defense against natural infections in mammalian systems. Second, these results raise the exciting yet challenging opportunity to develop therapeutic approaches employing RNAi.

Is RNA silencing an antiviral system? Even though synthetic siRNA transfected into mammalian cells can inhibit viral replication, it is not yet clear whether RNA silencing plays a physiological role as an antiviral system. For instance, it is unclear whether RNA silencing can be elicited naturally during viral infection. Since the presumably natural precursors of siRNAs, long dsRNA intermediates of RNA replication, seem to cause a general translation shutoff in mammalian cells, it is not yet clear how specific RNAi responses could be initiated during the course of a natural infection. Although the recent findings using siRNAs discussed here represent an important step toward determining the role and potential of RNAi as an antiviral mechanism, they are based on artificial experimental designs.

We propose that establishing RNAi as a natural antiviral defense in mammals will require addressing five critical issues that are schematically described in Fig. 2.

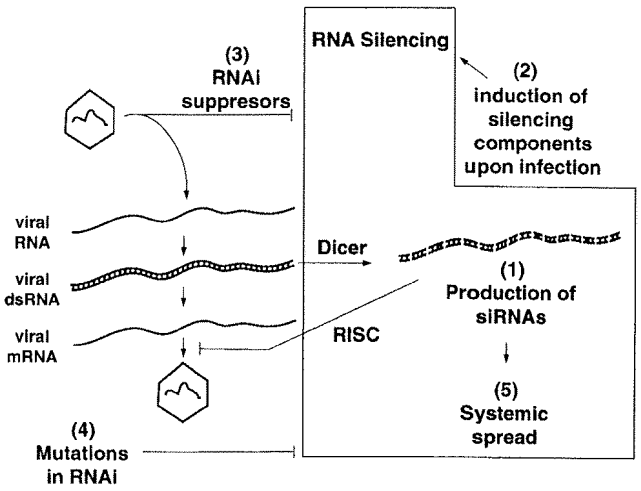

FIG. 2. Is RNA silencing a natural antiviral defense system in mammals? Establishing RNAi as a natural antiviral defense system in mammals will require addressing the five critical questions which are schematically presented in the figure. See text for details.

Issue 1: Are siRNAs generated during the course of a natural infection? To generate siRNAs from long dsRNA replication intermediates necessitates functional Dicer expression. Intriguingly, there is a difference in the response to dsRNA by embryonic stem cells and differentiated cell lines. Differentiated cell lines express PKR and RNase L, and apparently only low levels of Dicer (9), and thus dsRNA induces a general translation shutoff. In contrast, embryonic carcinoma cells process dsRNA to induce sequence-specific silencing (9). One possibility is that the antiviral/antitransposon function of RNA silencing is limited to embryos. Alternatively, certain cell types in the adult may be able to process and disseminate long dsRNA, while most other cells are only capable of reacting to fully processed siRNAs. However, since undifferentiated embryonic carcinoma cell lines are susceptible to viral infection (59), there may be unidentified regulatory mechanisms that control RNA silencing in embryonic cells in vivo. Otherwise, viruses may have found ways to overcome silencing or the RNAi machinery in embryonic cell lines may lack additional components required for antiviral function.

Issue 2: Are RNAi components upregulated during viral infection? The upregulation of RNAi components during viral infection would support the idea that this system plays a natural role in antiviral defense. The recent finding that porcine reproductive and respiratory syndrome virus infection induces upregulation of the RNA helicase RHIV-1 (70), a homologue of drh-1 helicase from *C. elegans* apparently required for dsRNA processing (58), may support this idea. However, it is possible, as suggested by the authors, that upregulation of RHIV-1 plays a role during virus replication rather than in antiviral defense. A related issue is the relationship between RNA silencing and the interferon system. Although it has been assumed that they are quite distinct and independent from each other, the relationship between these systems remains to be analyzed. Since many components of the RNAi system

appear to participate in micro-RNA processing (4), it will also be interesting to elucidate the overlap between RNAi and micro-RNA processing. Understanding the regulation and interplay of these two systems, especially in a context of a viral infection may have important consequences for the adaptation of RNAi for therapeutic purposes.

Issue 3: Have viruses evolved mechanisms to suppress or escape an RNAi response? Two types of mechanisms of RNAi evasion can be envisioned. First, as mentioned before, it is possible that viruses protect their RNAs by sequestering them in viral particles or replication complexes. However, because viruses need to express their genomic information by translating their RNAs and since mRNAs are susceptible to RNAi, it is possible that the RNA silencing machinery can target and reduce the expression of viral protein, thus inhibiting viral replication. Assuming the viral dsRNA intermediates are shielded from Dicer, how is the RNAi response initiated? One possibility is that dsRNA leaks out from the shielded compartment inside the infected cells or even from the remnants of cells lysed by the virus. A second type of evasion mechanism could rely on virus-encoded proteins that directly inhibit specific steps of RNA silencing. So far, there have been no reports of such activities encoded by mammalian viruses, but as the study of RNAi in mammalian cells develops, it is likely that information about virus-encoded RNAi suppressors will be obtained.

Issue 4: Do mutations or deletions in mammalian homologues of RNAi components render cells or animals more susceptible to viral infection? Both genetic and biochemical approaches have already identified several key components of the mammalian RNAi machinery (including Dicer and the RISC) (8, 41, 24), and more are likely to emerge in the near future. It is possible that knockouts of genes encoding RNA silencing components will be lethal, considering that they may participate in several RNA-based gene expression regulatory systems, as shown for DICER in micro-RNA processing (4). However, it may be possible to obtain cell lines deficient in specific components of the RNA silencing machinery to address whether defects in the RNAi pathway increase susceptibility to virus infection.

Issue 5: Can RNAi in one infected cell trigger a systemic antiviral response? In *C. elegans*, the injection of dsRNA in one region of the worm triggers its spread to many different tissues, including the gonads (19). It is not known how the dsRNA exits the cell in which it was originally produced, how it is transported through the organism, and how it is taken up by distant target cells. It will thus be important to address whether RNA silencing in mammals also induces a systemic response.

Is it possible to manipulate the RNAi system to develop therapeutic approaches? Present data has generated much hope for the use of RNAi as a novel antiviral therapy. However, establishing RNAi as a viable therapeutic approach requires resolving at least three major issues.

(i) Persistence of the RNAi inhibitory effect. As mentioned above, an inhibitory effect in cell culture is observed for only a few days after transfection of siRNAs. This relatively short duration of the RNA inhibitory effect has been circumvented by constitutively expressing shRNAs from RNA polymerase III promoters, with transcription initiated and terminated at precise sites (for a review, see reference 61) or as polymerase II transcripts (68, 69). Although shRNAs have not yet been extensively employed, it appears that the system is effective in a variety of cell types. Intriguingly, it appears that Dicer is responsible for processing shRNA into siRNAs (47), suggesting that this artificial system employs the RNAi processing apparatus to channel the shRNA into the correct pathway. Similar approaches may be extended to solve the persistence issue.

(ii) Delivery. A major problem to be addressed is how to efficiently deliver the shRNA-expressing plasmids and how to target specific cell types. Perhaps a reasonable approach will be to employ viral vectors. Initial reports indicate that retroviral (7, 17) and adenoviral (68) vectors are capable of carrying shRNAs and inducing RNAi in target cells. Nonetheless, it is expected that this approach will encounter benefits and limitations similar to those observed in gene therapy methodologies.

(iii) Viral escape. As mentioned above, viruses are likely to evade any given siRNA by mutations of the target sequences. Therefore, it may be important to produce multiple siRNAs, focusing on the conserved regions of the viral genome. However, this strategy has not yet been evaluated and it is possible that viruses are able to find additional ways to evade a strategy based on targeting multiple sites in the viral genome. An alternative method to circumvent the high rate of viral mutation may be to target a cellular protein required for viral replication. For example, depletion of CD4 using siRNAs has led to a decrease in the infectivity of HIV (45). These experiments suggest that targeting CCR5 (which is mutated in some individuals that are resistant to HIV infection [40]) may be a successful course in protection from AIDS. In a very recent report, CCR5 was targeted by siRNAs. Blocking CCR5 expression resulted in substantial protection for the lymphocyte populations susceptible to HIV-1 infection (49). However, the biological plasticity of viruses may find ways to overcome this strategy and, thus, targeting a cellular factor may not guarantee complete protection from viral infection (52, 60). Hence, it is possible that the genetic variability of RNA viruses may render targeting a single cellular factor ineffective. Therefore, targeting several host cell factors involved in viral replication may be required, an approach designed as an RNAi equivalent to the multi-drug antiretrovirus therapy HAART, whose success is based on the simultaneous targeting of multiple viral proteins.

In hindsight, one would expect researchers to have predicted the existence of a nucleic acid-based immune system earlier, by analogy to the protein-based immune system of mammals. After all, if life began as an RNA world it would have been appropriate to evolve an adaptive system that recognized foreign nucleic acids early on. The discovery of this highly sophisticated system stresses the advantages of being both adaptive and specific in combating invaders. It also stresses the fact that there is more to RNA silencing and virology in general than we can yet fathom. The years to come promise to bring interesting and unanticipated results regarding the mechanism and therapeutic applications of this fascinating system.

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ADDENDUM IN PROOF

While this paper was under review, the following articles dealing with siRNA application against an orthomyxovirus, a herpesvirus, and hepatitis C virus replicons appeared: Q. Ge, M. T. McManus, T. Nguyen, C. H. Shen, P. A. Sharp, H. N. Eisen, and J. Chen, Proc. Natl. Acad. Sci. USA **100:**2718–2723, 2003; Q. Jia and R. Sun, J. Virol. **77:**3301–3306, 2003; J. A. Wilson, S. Jayasena, A. Khvorova, S. Sabatinos, I. G. Rodrigue-Gerbais, S. Arya, F. Sarangi, M. Harris-Brandts, S. Beaulieu, and C. D. Richardson, Proc. Natl. Acad. Sci USA **100:**2783–2788, 2003; and S. B. Kapadia, A. Brideau-Andersen, and F. V. Chisari, Proc. Natl. Acad. Sci. USA **100:** 2014–2018, 2003.

REFERENCES

1. **Adelman, Z. N., C. D. Blair, J. O. Carlson, B. J. Beaty, and K. E. Olson.** 2001. Sindbis virus-induced silencing of dengue viruses in mosquitoes. Insect Mol. Biol. **10:**265–273.
2. **Adelman, Z. N., I. Sanchez-Vargas, E. A. Travanty, J. O. Carlson, B. J. Beaty, C. D. Blair, and K. E. Olson.** 2002. RNA silencing of dengue virus type 2 replication in transformed C6/36 mosquito cells transcribing an inverted-repeat RNA derived from the virus genome. J. Virol. **76:**12925–12933.
3. **Ahlquist, P.** 2002. RNA-dependent RNA polymerases, viruses, and RNA silencing. Science **296:**1270–1273.

3a. **Al-Kaff, N. S., S> N. Covey, M. M. Kreike, A. M. Page, R. Pinder, and P. J. Dale.** 1998. Transcriptional and posttranscriptional plant gene silencing in response to a pathogen. Science **279:**2113–2115.

4. **Ambros, V.** 2001. MicroRNAs: tiny regulators with great potential. Cell **107:**823–826.
5. **Anandalakshmi, R., G. J. Pruss, X. Ge, R. Marathe, A. C. Mallory, T. H. Smith, and V. B. Vance.** 1998. A viral suppressor of gene silencing in plants. Proc. Natl. Acad. Sci. USA **95:**13079–13084.
6. **Astier-Manifacier, S., and P. Cornuet.** 1971. RNA-dependent RNA polymerase in Chinese cabbage. Biochim. Biophys. Acta **232:**484–493.
7. **Barton, G. M., and R. Medzhitov.** 2002. Retroviral delivery of small interfering RNA into primary cells. Proc. Natl. Acad. Sci. USA **99:**14943–14945.
8. **Bernstein, E., A. A. Caudy, S. M. Hammond, and G. J. Hannon.** 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature **409:**363–366.
9. **Billy, E., V. Brondani, H. Zhang, U. Muller, and W. Filipowicz.** 2001. Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. Proc. Natl. Acad. Sci. USA **98:**14428–14433.
10. **Bitko, V., and S. Barik.** 2001. Phenotypic silencing of cytoplasmic genes using sequence-specific double-stranded short interfering RNA and its application in the reverse genetics of wild type negative-strand RNA viruses. BMC Microbiol. **1:**34.
11. **Brigneti, G., O. Voinnet, W. X. Li, L. H. Ji, S. W. Ding, and D. C. Baulcombe.** 1998. Viral pathogenicity determinants are suppressors of transgene silencing in Nicotiana benthamiana. EMBO J. **17:**6739–6746.
12. **Caplen, N. J., S. Parrish, F. Imani, A. Fire, and R. A. Morgan.** 2001. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. Proc. Natl. Acad. Sci. USA **98:**9742–9747.
13. **Capodici, J., K. Kariko, and D. Weissman.** 2002. Inhibition of HIV-1 Infection by small interfering RNA-mediated RNA interference. J. Immunol. **169:**5196–5201.
14. **Coburn, G. A., and B. R. Cullen.** 2002. Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. J. Virol. **76:**9225–9231.
15. **Colby, C., C. Jurale, and J. R. Kates.** 1971. Mechanism of synthesis of vaccinia virus double-stranded ribonucleic acid in vivo and in vitro. J. Virol. **7:**71–76.
16. **Dalmay, T., R. Horsefield, T. H. Braunstein, and D. C. Baulcombe.** 2001. SDE3 encodes an RNA helicase required for post-transcriptional gene silencing in Arabidopsis. EMBO J. **20:**2069–2078.
17. **Devroe, E., and P. A. Silver.** 2002. Retrovirus-delivered siRNA. BMC Biotechnol. **2:**15.

17a. **Dougherty, W. G., and T. D. Parks.** 1995. Transgene and gene suppression: telling us something new? Curr. Opin. Cell Biol. **7:**399–405.

18. **Fields, B. N., D. M. Knipe, and P. M. Howley.** 1996. Fields virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa.
19. **Fire, A.** 1999. RNA-triggered gene silencing. Trends Genet. **15:**358–363.
20. **Gaines, P. J., K. E. Olson, S. Higgs, A. M. Powers, B. J. Beaty, and C. D. Blair.** 1996. Pathogen-derived resistance to dengue type 2 virus in mosquito cells by expression of the premembrane coding region of the viral genome. J. Virol. **70:**2132–2137.
21. **Gitlin, L., S. Karelsky, and R. Andino.** 2002. Short interfering RNA confers intracellular antiviral immunity in human cells. Nature **418:**430–434.
22. **Guidotti, L. G., and F. V. Chisari.** 2001. Noncytolytic control of viral infections by the innate and adaptive immune response. Annu. Rev. Immunol. **19:**65–91.
23. **Guidotti, L. G., R. Rochford, J. Chung, M. Shapiro, R. Purcell, and F. V. Chisari.** 1999. Viral clearance without destruction of infected cells during acute HBV infection. Science **284:**825–829.
24. **Hammond, S. M., S. Boettcher, A. A. Caudy, R. Kobayashi, and G. J. Hannon.** 2001. Argonaute2, a link between genetic and biochemical analyses of RNAi. Science **293:**1146–1150.
25. **Hannon, G. J.** 2002. RNA interference. Nature **418:**244–251.
26. **Hu, W., C. Myers, J. Kilzer, S. Pfaff, and F. Bushman.** 2002. Inhibition of retroviral pathogenesis by RNA interference. Curr. Biol. **12:**1301.
27. **Hutvagner, G., and P. D. Zamore.** 2002. RNAi: nature abhors a double-strand. Curr. Opin. Genet. Dev. **12:**225–232.
28. **Jacque, J. M., K. Triques, and M. Stevenson.** 2002. Modulation of HIV-1 replication by RNA interference. Nature **418:**435–438.
29. **Jiang, M., and J. Milner.** 2002. Selective silencing of viral gene expression in HPV-positive human cervical carcinoma cells treated with siRNA, a primer of RNA interference. Oncogene **21:**6041–6048.
30. **Kasschau, K. D., and J. C. Carrington.** 1998. A counterdefensive strategy of plant viruses: suppression of posttranscriptional gene silencing. Cell **95:**461–470.
31. **Kennerdell, J. R., and R. W. Carthew.** 1998. Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. Cell **95:**1017–1026.
32. **Kimura, T., and D. E. Griffin.** 2000. The role of CD8$^+$ T cells and major histocompatibility complex class I expression in the central nervous system of mice infected with neurovirulent Sindbis virus. J. Virol. **74:**6117–6125.
33. **Kumagai, M. H., J. Donson, G. della-Cioppa, D. Harvey, K. Hanley, and L. K. Grill.** 1995. Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. Proc. Natl. Acad. Sci. USA **92:**1679–1683.
34. **Kumar, M., and G. G. Carmichael.** 1997. Nuclear antisense RNA induces extensive adenosine modifications and nuclear retention of target transcripts. Proc. Natl. Acad. Sci. USA **94:**3542–3547.
35. **Lee, N. S., T. Dohjima, G. Bauer, H. Li, M. J. Li, A. Ehsani, P. Salvaterra, and J. Rossi.** 2002. Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. Nat. Biotechnol. **20:**500–505.
36. **Leib, D. A., M. A. Machalek, B. R. Williams, R. H. Silverman, and H. W. Virgin.** 2000. Specific phenotypic restoration of an attenuated virus by knockout of a host resistance gene. Proc. Natl. Acad. Sci. USA **97:**6097–6101.
37. **Li, H. W., W. X. Li, and S. W. Ding.** 2002. Induction and suppression of RNA silencing by an animal virus. Science **296:**1319–1321.
38. **Lindbo, J. A., L. Silva-Rosales, W. M. Proebsting, and W. G. Dougherty.** 1993. Induction of a highly specific antiviral state in transgenic plants—implications for regulation of gene expression and virus resistance. Plant Cell **5:**1749–1759.
39. **Lipardi, C., Q. Wei, and B. M. Paterson.** 2001. RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. Cell **107:**297–307.
40. **Liu, R., W. A. Paxton, S. Choe, D. Ceradini, S. R. Martin, R. Horuk, M. E. MacDonald, H. Stuhlmann, R. A. Koup, and N. R. Landau.** 1996. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell **86:**367–377.
41. **Martinez, J., A. Patkaniowska, H. Urlaub, R. Luhrmann, and T. Tuschl.** 2002. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell **110:**563.
42. **Mourrain, P., C. Beclin, T. Elmayan, F. Feuerbach, C. Godon, J. B. Morel, D. Jouette, A. M. Lacombe, S. Nikic, N. Picault, K. Remoue, M. Sanial, T. A. Vo, and H. Vaucheret.** 2000. Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. Cell **101:** 533–542.
43. **Napoli, C., C. Lemieux, and R. Jorgensen.** 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. Plant Cell **2:**279–289.
44. **Ngo, H., C. Tschudi, K. Gull, and E. Ullu.** 1998. Double-stranded RNA induces mRNA degradation in Trypanosoma brucei. Proc. Natl. Acad. Sci. USA **95:**14687–14692.
45. **Novina, C. D., M. F. Murray, D. M. Dykxhoorn, P. J. Beresford, J. Riess, S. K. Lee, R. G. Collman, J. Lieberman, P. Shankar, and P. A. Sharp.** 2002. siRNA-directed inhibition of HIV-1 infection. Nat. Med. **8:**681–686.
46. **Olson, K. E., S. Higgs, P. J. Gaines, A. M. Powers, B. S. Davis, K. I. Kamrud, J. O. Carlson, C. D. Blair, and B. J. Beaty.** 1996. Genetically engineered resistance to dengue-2 virus transmission in mosquitoes. Science **272:**884–886.
47. **Paddison, P. J., A. A. Caudy, E. Bernstein, G. J. Hannon, and D. S. Conklin.** 2002. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev. **16:**948–958.
48. **Park, W. S., N. Miyano-Kurosaki, M. Hayafune, E. Nakajima, T. Matsuzaki,**

F. Shimada, and H. Takaku. 2002. Prevention of HIV-1 infection in human peripheral blood mononuclear cells by specific RNA interference. Nucleic Acids Res. **30:**4830–4835.

49. **Qin, X. F., D. S. An, I. S. Chen, and D. Baltimore.** 2003. Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. Proc. Natl. Acad. Sci. USA **100:**183–188.
50. **Randall, G., A. Grakoui, and C. M. Rice.** 2003. Clearance of replicating hepatitis C virus replicon RNAs in cell culture by small interfering RNAs. Proc. Natl. Acad. Sci. USA **100:**235–240.

50a. **Ratcliff, F. G., S. A. MacFarlane, and D. C. Baulcombe.** 1999. Gene silencing without DNA. RNA-mediated cross-protection between viruses. Plant Cell **11:**1207–1216.

51. **Schiebel, W., B. Haas, S. Marinkovic, A. Klanner, and H. L. Sanger.** 1993. RNA-directed RNA polymerase from tomato leaves. I. Purification and physical properties. J. Biol. Chem. **268:**11851–11857.
52. **Schols, D., J. A. Este, C. Cabrera, and E. De Clercq.** 1998. T-cell-line-tropic human immunodeficiency virus type 1 that is made resistant to stromal cell-derived factor 1α contains mutations in the envelope gp120 but does not show a switch in coreceptor use. J. Virol. **72:**4032–4037.
53. **Sequeira, L.** 1984. Cross-protection and induced resistance: their potential for plant disease control. Trends Biotechnol. **2:**25–29.
54. **Sijen, T., J. Fleenor, F. Simmer, K. L. Thijssen, S. Parrish, L. Timmons, R. H. Plasterk, and A. Fire.** 2001. On the role of RNA amplification in dsRNA-triggered gene silencing. Cell **107:**465–476.
55. **Stark, G. R., I. M. Kerr, B. R. Williams, R. H. Silverman, and R. D. Schreiber.** 1998. How cells respond to interferons. Annu. Rev. Biochem. **67:**227–264.
56. **Surabhi, R. M., and R. B. Gaynor.** 2002. RNA interference directed against viral and cellular targets inhibits human immunodeficiency virus type 1 replication. J. Virol. **76:**12963–12973.
57. **Svoboda, P., P. Stein, H. Hayashi, and R. M. Schultz.** 2000. Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference. Development **127:**4147–4156.
58. **Tabara, H., E. Yigit, H. Siomi, and C. C. Mello.** 2002. The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExH-box helicase to direct RNAi in C. elegans. Cell **109:**861–871.
59. **Teich, N. M., R. A. Weiss, G. R. Martin, and D. R. Lowy.** 1977. Virus infection of murine teratocarcinoma stem cell lines. Cell **12:**973–982.
60. **Trkola, A., S. E. Kuhmann, J. M. Strizki, E. Maxwell, T. Ketas, T. Morgan, P. Pugach, S. Xu, L. Wojcik, J. Tagat, A. Palani, S. Shapiro, J. W. Clader, S. McCombie, G. R. Reyes, B. M. Baroudy, and J. P. Moore.** 2002. HIV-1 escape from a small molecule, CCR5-specific entry inhibitor does not involve CXCR4 use. Proc. Natl. Acad. Sci. USA **99:**395–400.
61. **Tuschl, T.** 2002. Expanding small RNA interference. Nat. Biotechnol. **20:** 446–448.
62. **van der Krol, A. R., L. A. Mur, M. Beld, J. N. Mol, and A. R. Stuitje.** 1990. Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell **2:**291–299.
63. **Vance, V., and H. Vaucheret.** 2001. RNA silencing in plants–defense and counterdefense. Science **292:**2277–2280.
64. **Voinnet, O., Y. M. Pinto, and D. C. Baulcombe.** 1999. Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. Proc. Natl. Acad. Sci. USA **96:**14147–14152.
65. **Wassenegger, M., and T. Pelissier.** 1998. A model for RNA-mediated gene silencing in higher plants. Plant Mol. Biol. **37:**349–362.
66. **Waterhouse, P. M., M. W. Graham, and M. B. Wang.** 1998. Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc. Natl. Acad. Sci. USA **95:**13959–13964.
67. **Wianny, F., and M. Zernicka-Goetz.** 2000. Specific interference with gene function by double-stranded RNA in early mouse development. Nat. Cell. Biol. **2:**70–75.
68. **Xia, H., Q. Mao, H. L. Paulson, and B. L. Davidson.** 2002. siRNA-mediated gene silencing in vitro and in vivo. Nat. Biotechnol. **16:**16.
69. **Zeng, Y., E. J. Wagner, and B. R. Cullen.** 2002. Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. Mol. Cell **9:**1327–1333.
70. **Zhang, X., C. Wang, L. B. Schook, R. J. Hawken, and M. S. Rutherford.** 2000. An RNA helicase, RHIV-1, induced by porcine reproductive and respiratory syndrome virus (PRRSV) is mapped on porcine chromosome 10q13. Microb Pathog. **28:**267–278.

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siRNAs: a new wave of RNA-based therapeutics

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Introducing silence

In 1998, Fire *et al.*[1] made the startling discovery that double-stranded RNA (dsRNA) could induce a potent silencing effect on homologous genes in the nematode *Caenorhabditis elegans*. This technique, termed RNA interference (RNAi), has proved to be a powerful tool with which to dissect gene function in plants, *C. elegans* and *Drosophila*. Although RNAi is evolutionarily conserved among plants and animals, silencing of specific genes in mammalian cells has been difficult because of the induction of the interferon response by dsRNAs of ≥30 nt.[2,3] This non-specific response to dsRNA leads to global changes in cellular gene expression and apoptosis, masking any specific silencing effect by RNAi in mammalian cells. Recently, however, it was shown that potent and specific gene silencing could be achieved in human cells transfected with small interfering RNAs (siRNA) of 21–23 nt, a key intermediate in the RNAi pathway.[4] This landmark discovery by Tuschl and co-workers has led to routine RNA interference in mammalian cell culture and may have finally opened the door to tractable genetic analysis in mammalian cells.

Current models of RNAi suggest that the pathway involves a five-step process (for an excellent review see reference 2). First, dsRNAs (>26 bp) are cleaved by an RNase III-like enzyme, termed Dicer, to generate 21–23 nt fragments (step 1).[5–7] These short duplexed siRNAs have two unusual properties that are critical for their function: they contain 2 nt 3′-overhangs and 5′-phosphate groups.[8] It is presumed that an ATP-dependent RNA helicase recognizes these short duplexes and resolves the siRNA duplex into two single-stranded RNAs (step 2).[9] One strand is then incorporated into a high-molecular-weight protein complex termed RISC (RNA-induced silencing complex) (step 3),[9] where it serves as guide RNA to direct the cleavage of homologous RNA sequences by an as yet unidentified endonucleolytic component of RISC (step 4).[8,10] Finally, RISC is liberated from the cleaved mRNA and is recycled to perform multiple rounds of catalysis (step 5). This property of RISC is responsible for the potent nature of the silencing effect.

An intriguing facet of the RNAi pathway is the tight link between RNAi and developmental timing in *C. elegans*.[11] The enzyme Dicer is required for both induction of RNAi and processing of two small temporal RNAs (stRNAs), *lin*-4 and *let*-7 from ~70 nt structured precursor RNAs.[6,7] Interestingly, the 21 nt single-stranded stRNAs, also referred to as microRNAs (miRNAs), are identical in length to siRNAs; however, unlike siRNAs that trigger mRNA degradation, stRNAs bind to multiple sites within the 3′ UTRs of target mRNAs and inhibit their translation by a mechanism that remains poorly understood.[12] The finding that repressed mRNAs are associated with polysomes suggests that translation initiation is not affected.[12]

Additional observations have further strengthened the connection between the RNAi and miRNA pathways. Purification of miRNP complexes from HeLa extracts led to the isolation of >40 unique miRNAs.[13] The miRNP complex isolated from human cells is the same approximate size as *Drosophila* RISC (~500 kDa), and one member of the complex, eIF2C2, an orthologue of Argonaute-2, is a known component of *Drosophila* RISC.[14] Secondly, and more importantly, the recent discovery that the endogenous human *let*-7 miRNA not only directs mRNA cleavage but also co-purifies with human RISC,[15] implies that RISC mediates both silencing processes. The choice of RISC action is probably dependent on the degree of complementarity between the guide RNA and the target sequence, with mRNA cleavage activity requiring almost 100% complete base pairing between the siRNA and target sequence.[15] Moreover, the finding that at least one plant miRNA can function as an siRNA, directing the cleavage of an endogenous mRNA in *Arabidopsis*,[16] provides a clear demonstration that at least some miRNAs can enter the

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RNAi pathway. The discovery of this new class of RNA, with >200 distinct miRNAs now catalogued in nematodes,[17,18] fruit flies,[19] humans[13] and plants,[16,20] suggests that miRNAs and RNAi may play a more general role in regulating gene expression than hitherto imagined.

RNAi-mediated inhibition of viral replication

In plants, it has long been recognized that post-transcriptional gene silencing and RNAi play critical roles in genome surveillance, protecting the cell from inappropriate expression of repetitive sequences, transposable elements and viruses.[21] In fact, certain plant viruses, including potyviruses, potato virus X and cucumber mosaic virus, have evolved proteins that antagonize the RNAi pathway, providing some of the strongest evidence that RNAi can serve as an innate cellular antiviral mechanism (reviewed in 21 and references therein). The finding that an animal virus, flock house virus (a small, positive-strand RNA virus, belonging to the Novaviridae, that infects insect cells and is morphologically similar to some plant viruses), also encodes an RNAi antagonist (the B2 protein) and is subject to RNAi in *Drosophila* in the absence of B2 expression,[22] clearly demonstrates that the antiviral properties of the RNAi pathway are conserved between the plant and animal kingdoms. The potential ability to tap into this native antiviral pathway, as a therapeutic strategy to target viruses and viral gene expression, has generated great excitement among many researchers. Several initial studies, which test the potential application of synthetic siRNAs as antiviral agents, have shown significant promise. To date, RNAi has been used effectively to inhibit the replication of several different pathogenic viruses in culture, including: RSV (respiratory syncytial virus),[23] poliovirus[24] and HIV-1.[25–27] In the case of HIV-1, several specific mRNAs have been successfully targeted for siRNA-mediated silencing, including those that encode Gag, Pol, Vif and the small regulatory proteins Tat and Rev. These studies show that RNAi can effectively trigger the degradation of not only viral mRNAs, but also genomic RNAs at both the pre- and post-integration stages of the viral lifecycle.[25–27] In addition to targeting viruses directly, alternative strategies have employed siRNAs that silence the expression of essential host factors including Tsg101, required for vacuolar sorting and efficient budding of HIV-1 progeny,[28] and the chemokine receptor CCR5, required as a co-receptor for HIV-1 cell entry.[29]

One impediment to utilizing RNAi technology for therapeutic benefit in humans remains the development of efficient delivery systems for siRNAs. Previous methods relied on harsh lipid-based transfection reagents to introduce siRNAs into cells in culture and are either inefficient and/or unsuitable for use in animals. An additional caveat is that, unlike in plants and lower eukaryotes, RNAi-mediated gene silencing is not long lasting in mammals. In cell culture, gene silencing effects can disappear within three to four generations, with the targeted protein returning to normal levels quickly thereafter.[4] An enormous step forward in addressing these complications is the finding that natural or designed siRNAs and miRNAs can be expressed *in vivo*.[30–33] Short hairpin and pre-miRNA-based precursor RNAs can be transcribed from either RNA polymerase III (H1 or U6)[30,32] or RNA polymerase II promoters[33] *in vivo*, and processed by Dicer to release functional siRNAs. Importantly, siRNAs expressed from DNA templates can silence gene expression as effectively as exogenously introduced synthetic siRNAs.[30,31,33] As an extension of these studies, many groups have begun constructing the first generation of retroviral-,[34] adenoviral-[35] and lentiviral-based[36] gene therapy vectors that are capable of expressing siRNAs in a stable manner in virtually any cell and tissue type. Already progress has been made, with the demonstration that murine retroviral vectors expressing siRNAs directed against a mutant allele of the human K-Ras proto-oncogene have the ability to reverse tumorigenicity in mice.[34] The further development of these delivery systems will push siRNA technology quickly from the 'proof of principle' phase into animal studies of important human diseases.

Conclusions and future challenges

It is striking how rapidly the field has moved from an initial discovery phase to a stage where implementation of siRNA technology in a therapeutic setting appears not only foreseeable, but imminent. Currently, however, our understanding of the biological mechanisms underlying RNAi lags behind the movement to apply this technology to human diseases such as cancer and infectious diseases such as HIV-1 and hepatitis C virus. Clearly the objectives, in the short term, are to improve viral delivery systems with the goal of maximizing siRNA expression. Presumably, this would involve optimization of promoters and siRNA precursor design. At present, information pertaining to endogenous miRNA promoter usage and important sequence and RNA structural constraints for Dicer processing are entirely lacking. A better understanding of the fundamental biochemistry of the RNAi pathway would certainly lead to improved target site selection and better overall siRNA design.

The appearance of a resistant strain of poliovirus in cultures treated with polio-specific siRNAs[24] is a strong reminder that a single nucleotide mutation could render viruses immune to a single specific siRNA. Although it was concluded that the mutation was already present in the primary viral isolate, emergence of siRNA resistance is a major concern that will need to be addressed, particularly for viruses encoding error-prone polymerases such as HIV-1. Thus, in future, siRNA expression cassettes will require a higher degree of sophisti-

cation, probably encoding tandem arrays of highly expressed siRNAs that target several conserved viral sequences simultaneously.

Much remains to be accomplished, but RNAi will continue to play an important role in determining cellular gene function and shows a great deal of promise as a therapeutic agent. The next few years of research will indicate whether RNAi technology will realize its potential as the 'next wave of therapeutic molecules'.[3]

References

1. Fire, A., Xu, S.-Q., Montgomery, M. K., Kostas, S. A., Driver, S. E. & Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–11.

2. Hutvágner, G. & Zamore, P. D. (2002). RNAi: nature abhors a double strand. *Current Opinion in Genetics and Development* **12**, 225–32.

3. McMannus, M. T. & Sharp, P. A. (2002). Gene silencing in mammals by small interfering RNAs. *Nature Reviews Genetics* **10**, 737–47.

4. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. & Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–8.

5. Bernstein, E., Caudy, A. A., Hammond, S. M. & Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363–6.

6. Ketting, R. F., Fischer, S. E., Bernstein, E., Sijen, T., Hannon, G. J. & Plasterk, R. H. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. *Genes and Development* **15**, 2654–9.

7. Knight, S. W. & Bass, B. L. (2001). A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in *Caenorhabditis elegans*. *Science* **293**, 2269–71.

8. Elbashir, S. M., Lendeckel, W. & Tuschl, T. (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes and Development* **15**, 188–200.

9. Nykänen, A., Haley, B. & Zamore, P. D. (2001). ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* **107**, 309–21.

10. Hammond, S. M., Bernstein, E., Beach, D. & Hannon, G. J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* **404**, 293–6.

11. Grishok, A., Pasquinelli, A. E., Conte, D., Li, N., Parrish, S., Ha, I. *et al.* (2001). Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. *Cell* **106**, 23–34.

12. Ambros, V. (2000). Control of developmental timing in *Caenorhabditis elegans*. *Current Opinion in Genetics and Development* **10**, 428–33.

13. Mourelatos, Z., Dostie, J., Paushkin, S., Sharma, A., Charroux, B., Abel, L. *et al.* (2002). miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. *Genes and Development* **16**, 720–8.

14. Hammond, S. M., Boettcher, S., Caudy, A. A., Kobayashi, R. & Hannon, G. J. (2001). Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* **293**, 1146–50.

15. Hutvágner, G. & Zamore, P. D. (2002). A microRNA in a multiple turnover RNAi complex. *Science* **292**, 2056–60.

16. Tang, G., Reinhart, B. J., Bartel, D. P. & Zamore, P. D. (2003). A biochemical framework for RNA silencing in plants. *Genes and Development* **17**, 49–63.

17. Lau, N. C., Lim, L. P., Weinstein, E. G. & Bartel, D. P. (2001). An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science* **294**, 858–62.

18. Lee, R. C. & Ambros, V. (2001). An extensive class of small RNAs in *Caenorhabditis elegans*. *Science* **294**, 862–4.

19. Lagos-Quintana, M., Rauhut, R., Lendeckel, W. & Tuschl, T. (2002). Identification of novel genes coding for small expressed RNAs. *Science* **294**, 853–8.

20. Reinhart, B. J., Weinstein, E. G., Rhoades, M. W., Bartel, B. & Bartel, D. P. (2002). MicroRNAs in plants. *Genes and Development* **16**, 1616–26.

21. Li, W. X. & Ding, S. W. (2001). Viral suppressors of RNA silencing. *Current Opinion in Biotechnology* **12**, 151–4.

22. Li, H., Li, W. X. & Ding, S. W. (2002). Induction and suppression of RNA silencing by an animal virus. *Science* **296**, 1319–21.

23. Bitko, V. & Barik, S. (2001). Phenotypic silencing of cytoplasmic genes using sequence-specific double-stranded short interfering RNA and its application in the reverse genetics of wild type negative-strand RNA viruses. *BMC Microbiology* **1**, 34–46.

24. Gitlin, L., Karelsky, S. & Andino, R. (2002). Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature* **418**, 430–4.

25. Coburn, G. A. & Cullen, B. R. (2002). Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. *Journal of Virology* **76**, 9225–31.

26. Jacque, J. M., Triques, K. & Stevenson, M. (2002). Modulation of HIV-1 replication by RNA interference. *Nature* **418**, 435–8.

27. Novina, C. D., Murray, M. F., Dykxhoorn, D. M., Beresford, P. J., Riess, J., Lee, S. K. *et al.* (2002). siRNA-directed inhibition of HIV-1 infection. *Nature Medicine* **8**, 681–6.

28. Garrus, J. E., von Schwedler, U. K., Pornillos, O. W., Morham, S. G., Zavitz, K. H., Wang, H. E. *et al.* (2001). Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. *Cell* **107**, 55–65.

29. Martinez, M. A., Gutierrez, A., Armand-Ugon, M., Blanco, J., Parera, M., Gomez, J. *et al.* (2002). Suppression of chemokine receptor expression by RNA interference allows for inhibition of HIV-1 replication. *AIDS* **16**, 2385–90.

30. Brummelkamp, T. R., Bernards, R. & Agami, R. A. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**, 550–3.

31. Paddison, P. J., Caudy, A. A., Bernstein, E., Hannon, G. J. & Conklin, D. S. (2002). Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes and Development* **16**, 948–58.

32. Paul, C. P., Good, P. D., Winer, I. & Engelke, D. R. (2002). Effective expression of small interfering RNA in human cells. *Nature Biotechnology* **20**, 505–8.

33. Zeng, Y., Wagner, E. J. & Cullen, B. R. (2002). Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. *Molecular Cell* **9**, 1327–33.

34. Brummelkamp, T. R., Bernards, R. & Agami, R. A. (2002). Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell* **2**, 243–7.

35. Xia, H., Mao, Q., Paulson, H. L. & Davidson, B. L. (2002). siRNA-mediated gene silencing *in vitro* and *in vivo*. *Nature Biotechnology* **20**, 1006–10.

36. Qin, X.-F., An, D. S., Chen, I. S. & Baltimore, D. (2003). Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. *Proceedings of the National Academy of Sciences, USA* **100**, 183–8.

Interfering with disease: opportunities and roadblocks to harnessing RNA interference

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RNA interference (RNAi) is an evolutionarily conserved mechanism for silencing gene expression by targeted degradation of mRNA. Short double-stranded RNAs, known as small interfering RNAs (siRNA), are incorporated into an RNA-induced silencing complex that directs degradation of RNA containing a homologous sequence. RNAi has been shown to work in mammalian cells, and can inhibit viral infection and control tumor cell growth *in vitro*. Recently, it has been shown that intravenous injection of siRNA or of plasmids expressing sequences processed to siRNA can protect mice from autoimmune and viral hepatitis. RNAi could provide an exciting new therapeutic modality for treating infection, cancer, neurodegenerative disease and other illnesses.

RNA interference (RNAi) is an ancient defense strategy that protects plants and lower invertebrates from viral infection and genomic damage by insertable genetic elements [1–5]. RNAi silences gene expression in a sequence-specific manner by cleaving mRNAs containing short sequences – 19 or more nucleotides in length – that are closely homologous to a target sequence. Two years ago, Sayda Elbashir and colleagues sparked a revolution in research when they demonstrated that RNAi operates in mammalian cells [6]. Researchers then moved rapidly to apply this new technique to understand mammalian gene function and to block gene expression *in vitro* [7]. A few studies have recently provided the proof of principle that RNAi can be used to protect mice from autoimmune and viral hepatitis. In this review, we will discuss the promise and hurdles in harnessing RNAi for human therapy and some of the clinical situations in which this novel therapy might be first applied.

RNAi was initially described in plants and in *Caenorhabditis elegans*, when it was discovered that double-stranded RNA (dsRNA) was much more potent at silencing gene expression than either sense or antisense single-stranded RNA (ssRNA) [1–4]. Feeding worms with short dsRNA homologous to a sequence in an endogenous gene caused a dramatic and specific reduction of gene expression [8]. In parallel, the phenomena of co-suppression of endogenous gene expression and the antiviral response in plants were understood to be due to post-transcriptional gene silencing of homologous sequences [9–11]. In the past few years, it has become clear that similar RNAi mechanisms exist in plants, fungi and throughout the animal kingdom, from worms and flies to mammals. RNAi works through the targeted degradation of mRNAs containing homologous (and in most cases identical) sequences to introduced short oligonucleotides (Fig. 1). In some organisms other than mammals, silencing can be amplified and spreads to target neighboring sequences via a process that involves an RNA-dependent RNA polymerase [12,13]. RNAi can also occur

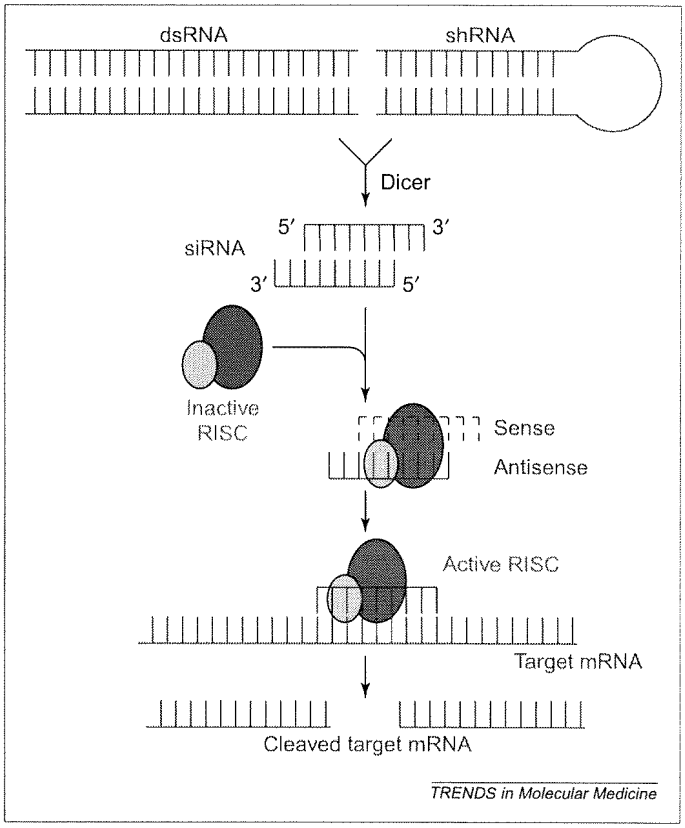

Fig. 1. Mechanism of RNA interference. Double-stranded (ds) RNA are processed by Dicer, in an ATP-dependent process, to produce small interfering RNAs (siRNA) of ~21–23 nucleotides in length with two-nucleotide overhangs at each end. Short hairpin (sh) RNAs, either produced endogenously or expressed from viral vectors, are also processed by Dicer into siRNA. An ATP-dependent helicase is required to unwind the dsRNA, allowing one strand to bind to the RNA-induced silencing complex (RISC). Binding of the antisense RNA strand activates the RISC to cleave mRNAs containing a homologous sequence.

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through additional mechanisms, which are just beginning to be described in mammalian cells [14]. These include some that work through changes in DNA methylation and chromatin structure induced by homologous dsRNAs, and others that work not through mRNA degradation but via translational arrest of mRNAs encoding closely homologous sequences [15–19]. These latter mechanisms use endogenous microRNAs (miRNA), which might be particularly important in regulating gene expression during development. miRNAs are often dsRNAs with imperfect pairing.

Intracellular generation of small interfering RNAs

In plants, small dsRNAs, complementary to both strands of the silenced gene, are generated from a longer dsRNA precursor [20]. RNAs of 21–22 nucleotides in length mediate RNAi, whereas longer sequences – 24 nucleotides in length – have been implicated in chromosomal silencing [21–23]. Processing of dsRNA to small interfering RNAs (siRNA) also occurs in *Drosophila* embryo and somatic cell extracts [24–27]. These endogenously generated siRNA have a unique dsRNA structure; each strand has a free 5′-phosphate end followed by 19 base pairs (bp) of paired RNA with a two-nucleotide unpaired overhang at the 3′ end (Fig. 1) [24,28]. The search for the enzyme that processes longer dsRNA into siRNA focused on the RNase III family of ribonucleases, whose substrates are duplex RNAs and whose products contain such 5′ ends. A class of evolutionarily conserved RNase-III-like enzymes, containing RNA helicase activity and sharing a common PAZ (piwi–argonaute–zwille) domain, has now been identified in plants and diverse animal species, and has been shown to process dsRNA into siRNA [29–31]. The siRNA-generating enzyme is called Dicer and is believed to act as a dimer. The RNAi machinery might also have other essential cellular functions; for example, deletion of RNAi genes in fission yeast interferes with proper segregation of chromosomes during cell division and maintenance of transcriptionally silenced heterochromatin [32–34]. Participation in important cellular functions could explain why there are no known examples of naturally occurring animal cells incapable of RNAi.

Mechanism of post-transcriptional gene silencing

The mechanism of gene silencing induced by siRNA, elucidated in plants, worms and flies, has only just begun to be described in mammalian cells. Initially in worms, RNAi was shown to act after transcription [35]. Using *Drosophila* embryo lysates, Phillip Sharp and colleagues then showed that silencing occurred via the destabilization of mRNA and suggested that dsRNA might trigger the assembly of a nuclease complex to target homologous RNAs for degradation [36]. The current model suggests that duplex siRNA are first unwound using an ATP-dependent helicase and then one strand of the siRNA is incorporated into a large precursor RNA-induced silencing complex (RISC) [27,37]. The activated RISC contains a single strand of RNA, a protein homologous to the product of the *Argonaute* gene family in plants (*rde-1* in *C. elegans*, *AGO2* in *Drosophila*, *GERp95* in human cells), and other uncharacterized factors, possibly including the unknown endonuclease responsible for cleaving the target RNA [37–40]. When the RNA in the RISC complex is complementary to a sequence in a target RNA, the RISC complex guides RNA cleavage in the middle of the homologous sequence.

In mammalian cells, the introduction of dsRNA > 30 nucleotides in length into the cytosol activates a programme of global gene suppression by translational arrest, by activating the antiviral interferon (IFN) response [41–45]. Long dsRNA, produced in the life cycle of most viruses, activates the dsRNA-dependent protein kinase PKR, which phosphorylates the α-chain of the translation elongation αinitiation factor EIF2α, blocking all protein synthesis. As part of the antiviral response, activated PKR also induces IFN production and activates RNaseL to degrade RNA. However, because siRNA are only 21–25 nucleotides in length, they do not efficiently activate PKR or IFN production, and hence their silencing effects are exquisitely sequence-specific [28,46].

Gene silencing using RNAi in mammalian cells

Although RNAi is an important antiviral defense in plants, there is currently no evidence that the endogenous antiviral response in mammals uses RNAi. However, the description of RNAi in mammals is very recent. Viral inhibitors of RNAi have been described in a few plant and animal viruses [47], so it would not be surprising if some viruses that infect mammals and produce a dsRNA intermediate in the cytoplasm have devised self-protective strategies that involve inhibition of RNAi.

Delivery or expression of siRNA in mammalian cells has now been used to silence a wide range of genes in a variety of cell types [7]. These studies have included proof-of-concept studies to silence reporter genes (those encoding green fluorescent protein and luciferase) [6,48–56] and studies to knockdown gene expression as a rapid probe for gene function (e.g. those encoding tubulin, DNA methyltransferase, lamin A, cadherin, Cdc20 and Cdk2) [6,52,54,57]. An ever-increasing number of reports have probed potential therapeutic uses. For example, RNAi has been shown to work *in vitro* in a variety of conditions: (1) against cancer, by silencing *Bcr–Abl*, mutated *Ras*, human papillomavirus (HPV) E6 and E7, activating transcription factor 2, or overexpressed proto-oncogenes such as *Bcl-2* [58–62]; (2) against autoimmune diseases, by silencing Fas and caspase-8 [63,64]; against neurodegenerative diseases, by silencing polyglutamine sequences [65]; and (4) against viral infections with diverse replication strategies, including respiratory syncytial virus, poliovirus, hepatitis B (HBV), hepatitis C (HCV), HIV, HPV and influenza [66–77]. Even genes that are highly transcribed, such as those encoding lamin A and tubulin, have been effectively silenced. However, silencing is typically incomplete – a 'knockdown' rather than a 'knockout' – with residual gene expression usually at least a few per cent above baseline. Some residual gene expression might be due to untransfected cells in these studies. The studies on HIV successfully targeted a variety of viral genes (*gag*, *rev*, *tat*), as well as the viral long terminal repeat and host cellular receptors (CD4 and CCR5). In rapidly dividing transformed cells, silencing by transfected siRNA lasts for less

than a week, but transduced macrophages are effectively silenced for at least three weeks, at least for some targeted genes [78]. siRNA directed against HIV p24 block HIV production only if given within a week of infection, but provide sustained inhibition for at least three weeks in already infected cells.

Specificity of gene silencing

In the *Ras* study, it was possible to target mutated *Ras* without affecting unmutated *Ras*, demonstrating the exquisite specificity of RNAi as a therapeutic tool [58]. However, this specificity could pose problems for therapeutic uses in viral infection, where viral escape from RNAi selection in poliovirus has already been demonstrated [73], although the resistant virus was already present in the infecting inoculum in this study. One way around viral escape is to target more than one gene, either in the virus or by targeting both host and viral genes [68,78,79]. Moreover, the RNAi mechanism can tolerate some sequence mismatches, particularly away from the middle cleavage site [28]. However, this raises some questions about whether nonspecific silencing of partially homologous genes will present a problem for therapeutic use. In fact, a recent *in vitro* study showed that some genes with incomplete homology could be partially silenced, an effect that was more pronounced at higher concentrations of siRNA [80].

Vectored delivery of siRNA

Although these early studies were carried out by transfecting chemically synthesized siRNAs, more recently, dsRNAs have been expressed from stem–loop structures encoded by plasmids, retroviruses and lentiviruses [48,51–54,58,67,81–84]. These vectors provide a means for *in vivo* transduction of a variety of cells, including non-dividing cells, and open up a range of possible therapeutic uses for RNAi. The expression plasmids generally use a Pol III promoter and express sense and antisense strands separately or as a hairpin structure [48,51–54,58,67,81,82]. In cells, Dicer processes the short hairpin (sh) RNA [sense strand – short (~5–9 bp) loop – antisense strand; or alternatively, antisense strand – loop – sense strand) to an effective siRNA (Fig. 1). Sequences of siRNA expressed from retroviral and lentiviral vectors have been shown to silence expression effectively in a variety of primary cells [including embryonic stem (ES) cells, lymphocytes, macrophages and dendritic cells] [83,84]. Silencing can be controlled by engineering inducible expression of stem–loops [85]. Transfected or infected ES cells have already been used to produce transgenic mice with constitutive or inducible expression of siRNA [83]. However, infection with viral vectors could activate antiviral or inflammatory responses in some cells and interfere with the intended specificity. In particular, a recent study showed that shRNA vectors encoding siRNA, too short to induce an IFN response on their own, can nonetheless induce the expression of genes participating in the IFN response [80].

In vivo protection from hepatitis using RNAi

Although most of these studies have been performed *in vitro*, often with co-transfection protocols, which might not mimic natural conditions, a few recent studies have demonstrated *in vivo* silencing using RNAi. For example, rapid injection of duplex siRNA in a large volume (~1 ml) into the tail vein of mice effectively silenced co-injected reporter gene expression [55,56]. However, hydrodynamic injections are difficult to perform reproducibly in mice and require the rapid injection of 10–20% of the mouse blood volume. Therefore, it is unlikely that this method could be readily scaled up for clinical use. In the mouse experiments, the reporter gene was efficiently expressed only in the liver and a few other organs, and hence silencing was also demonstrated only in these organs. Hydrodynamically injected duplex siRNA directed against *Fas* were efficiently taken up by mouse hepatocytes (88%), and effectively silenced endogenous gene expression for ten days without diminution [63]. *In vivo* silencing in hepatocytes began to wane by 14 days and disappeared by 21 days. Mice with silenced *Fas* expression were protected from hepatic destruction and death in two models of autoimmune hepatitis (Fig. 2). The livers of *Fas*-siRNA-treated mice, but not of control mice, were dramatically

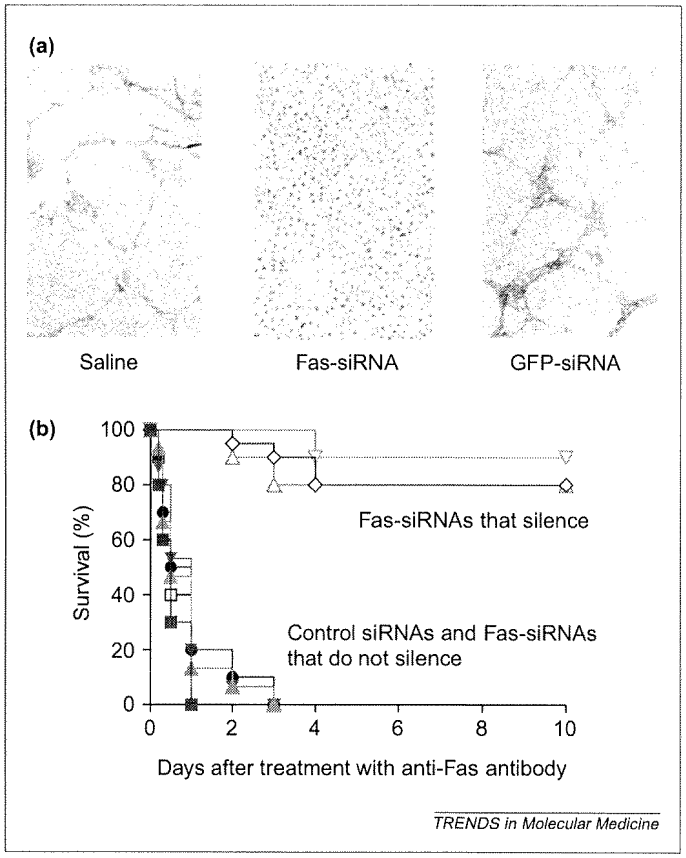

Fig. 2. *In vivo* protection from autoimmune hepatitis after high-pressure intravenous injection of small interfering RNAs (siRNA) targeting the apoptosis mediator, Fas. (a) Livers from mice given six weekly injections of concanavalin A were spared from fibrotic damage when Fas-siRNA was administered after the second and fourth injections (center panel). Injection of control saline solution (left panel) or of siRNA targeting the unrelated gene, green fluorescent protein (*GFP*) (right panel), did not prevent fibrotic damage. Original magnification × 100 for all panels. (b) In a more fulminant hepatitis model, induced by intraperitoneal injection of anti-Fas antibody, most mice pretreated with Fas-siRNA were protected from death, whereas all control mice died. Adapted from Ref. [63].

protected *in vivo* from treatment with concanavalin A (ConA) or anti-Fas antibody. In the former case, injections of siRNA could provide protection from hepatic fibrosis even when they were initiated after the second of six weekly injections of ConA. Anti-Fas siRNA protected mice from death induced by anti-Fas antibody; all control mice died within three days but 82% of *Fas*-siRNA-treated mice survived and had normal liver histology ten days later. Many forms of fulminant hepatitis, irrespective of etiology (viral, autoimmune or transplant rejection), are mediated by engaging the Fas receptor on hepatocytes, and hence silencing *Fas* expression or more distal mediators of apoptosis could be a useful strategy for treating hepatitis more generally. In fact, a recent study, silencing caspase-8 (activated downstream after Fas-receptor engagement), showed protection from both autoimmune and adenoviral hepatitis [64]. In addition, co-transfection by hydrodynamic injection in mice of an HBV plasmid [86] and a plasmid expressing shRNA targeting HBV sequences, processed to siRNA within cells, reduced HBV production and infection of liver cells [76]. Therefore, the potential of RNAi for treating hepatitis by targeting both apoptotic and viral genes has already been demonstrated *in vivo*.

Moving siRNA therapy from mice to people

Although hydrodynamic delivery of double-stranded siRNA can induce an effective silenced state in the livers of mice, it is unlikely that this strategy can be scaled up to humans because the introduction of duplex siRNA requires transiently generating high local intravascular pressure. In mice, siRNA are injected by bolus into the tail vein in a volume of ~10–20% of the mouse circulating blood volume. Although such a method could presumably be adapted to transduce selected tissues via regional catheterization [63,64] or to deliver siRNA pre-operatively into transplanted organs, it is unlikely to be possible to adapt this protocol safely for systemic human treatment.

Although dsRNA is resistant to most RNases, the intravenous half-life of unmodified duplex siRNA is very short, because the small molecules are degraded by endogenous nucleases and are quickly filtered through the kidneys. Therefore, duplex siRNA will need to be chemically modified for use as an injectable drug.

The usefulness of RNAi for therapy might be enhanced by targeted delivery to particular cells (such as virus-infected cells or tumors) or tissues, to prevent unwanted effects on normal host tissues. Targeted delivery can be achieved either by local administration (such as to the skin, mucosa or eye) or by injection via catheterization of the vascular supply to particular organs. In principle, chemically modified siRNA could also be covalently linked to ligands for cell-surface receptors, enabling delivery only to cells bearing these receptors. However, targeted delivery of duplex siRNA to induce a silenced state remains to be demonstrated. Similarly, viral vectors could theoretically be targeted by incorporating envelope genes for specific cell delivery.

Delivery strategies for therapy

Several strategies that have been developed to stabilize antisense oligonucleotides and ribozymes, including capping the 5′ ends, modifying the ribose sugars or substituting phosphorothioates, can stabilize siRNA without toxicity and might not jeopardize their ability to target mRNAs for degradation [87–89]. Moreover, some modifications of the sense strand, particularly at the 3′ end, are thought not to interfere with gene silencing [90]. Covalently coupling antibodies or ligands for cell-surface receptors to the 3′ end of the sense strand should provide a method for targeting siRNA specifically to cells or tissues of therapeutic interest. Covalent or even non-covalent linkage to basic peptides that deliver oligonucleotides into cells, or incorporation of siRNA into liposomes, could also provide a universal delivery mechanism [91,92]. Once inside the cell, duplex siRNA are expected to induce a silenced state that will last for 1–3 weeks (at least for cells that are not continuously undergoing cell division). This could be an ideal therapeutic window for many chronic diseases: long enough to require treatment only every few weeks or monthly, and short enough not to raise concerns about the long-term toxicity of permanently silencing a gene. However, introducing oligonucleotides into some non-dividing cells, such as lymphocytes or hematopoietic stem cells, is notoriously difficult and could require special strategies or delivery via viral vectors.

In addition to delivering siRNA as a drug, there is also considerable interest in delivering siRNA expressed from plasmids or viral vectors [48,51–54,58,67,76,79,81–84]. These approaches have all the benefits and drawbacks of gene therapy. The benefits are potential life-long expression of the siRNA, as a result of stable integration of the plasmid into genomic DNA, and the ability to target non-dividing cells such as stem cells, lymphocytes and neurons. The disadvantages are the low proportion of transduced cells and rapid loss of gene expression using current gene therapy methods, the danger of oncogenic transformation from insertional mutagenesis, and the danger of unanticipated toxicity from long-term silencing of human genes.

Therapeutic targets

Any gene is a potential target for silencing by RNAi, and hence the therapeutic possibilities are endless. This fact, coupled with the specificity and potency of silencing by siRNAs (~1000-fold more active on a molar basis than antisense oligonucleotides [93]), has caused some workers to tout siRNA as potentially the biggest revolution in therapeutics since the development of specific antibodies. Moreover, siRNA are likely to be much cheaper to manufacture and administer than antibody therapies. Therapeutic strategies for siRNA are based on silencing either 'nonessential' wild-type genes linked to disease or silencing mutated genes in which the mutation causes disease. Being able to deliver siRNA to specific cellular targets broadens the list of genes that can be silenced without inducing toxicity. Targeting mutated sequences is more costly and difficult to execute, because the therapy needs to be customized to the patient by determining the individual gene mutation and then synthesizing the homologous siRNA. The *in vitro* translational studies of RNAi carried out to date have focused mainly on viral infection and cancer, and these are likely to be the areas of early therapeutic efforts.

Viruses and other infectious agents

Although RNAi is an ancient antiviral defense in plants, it is unknown whether it plays any role in the natural defense of mammalian cells against viruses. Nonetheless, it seemed logical to try to harness this mechanism for the control of viral infection, particularly given that the silencing of viral genes is unlikely to have adverse consequences for the host. In searching for sequences to target, those that lack homology to human genes are used. RNAi could be used to treat acute viral infections, such as influenza and severe acute respiratory syndrome, which cause major morbidity and death, or to treat chronic infections that eventually progress. The chronic infections that cause the most morbidity throughout the world are HBV, hepatitis C (HCV) and HIV. Other intracellular pathogens, such as *Mycobacterium tuberculosis*, might also be amenable to RNAi. These are likely to be among the first therapeutic targets investigated.

Attractive viral targets are viral genes that are essential for replication, and sequences that are relatively conserved between viral strains, for which mutation would result in a less-robust infectious agent. Host genes that are required for viral entry or that play an essential role in the viral life cycle are also potentially good targets, providing that they are not required for survival of the cell. For RNA viruses, such as HIV or HCV, any region of the viral genome, including non-transcribed regions and regulatory genes, can also be targeted. It is possible that the RNAi machinery could be a limiting factor in mammalian cells, restricting the number of sequences that can be successfully targeted at the same time within a cell. Nonetheless, to minimize the risk of viral escape mutations undermining RNAi, the simultaneous targeting of several viral and/or host genes that are important at different stages in the viral life cycle might be a useful strategy. Moreover, synergistic enhancement of viral suppression has been demonstrated by targeting more than one gene [78].

Although bacteria are not amenable to silencing by siRNA, because they mainly replicate outside of cells and lack the RNAi machinery, it might be possible to reduce morbidity and mortality from life-threatening bacterial infections by silencing genes involved in those aspects of the stereotypical host immune response that lead to adverse consequences. For example, reducing the expression of pro-inflammatory cytokines, such as interleukin-1 (IL-1) or tumor necrosis factor α (TNF-α), might lessen the risk of septic shock, without jeopardizing the development of protective immunity. Such treatments would complement antibiotic therapies and could be instituted even before an infectious agent has been identified. In fact, in a recent study in mice, sepsis induced by treatment with lipopolysaccharide was blocked by injecting liposomes containing duplex siRNA targeting TNF-α [94].

Cancer

Several *in vitro* studies have already demonstrated the potential use of RNAi for treating cancers [58–61]. Gene targets that have been shown to slow tumor growth include the products of genes that are characteristically mutated generically or in specific cancers – either as translocations such as *Bcr–Abl* in chronic myeloid leukemia, or as point mutations such as *Ras* – to produce constitutively active oncogenes. The oncogenic potential of *Ras* mutation is atypical in that the changing of a single amino acid renders the molecule constitutively active and oncogenic, and hence a small number of different mutations are seen repeatedly in many cancers. As a result, targeting *Ras* would not need require a customized therapy for each tumor. However, although most of the other types of cancer mutations, such as translocations, occur in fairly well-defined locations, they are nonetheless too diverse to be targeted without sequencing the mutated gene for each tumor that needs to be treated. However, targeting the downstream sequence of a translocated gene, such as an *Abl* sequence in *Bcr–Abl*, might be an effective therapy, particularly if the tumor cell could be transfected selectively *in vivo*.

However, there are several genes that are not mutated in cancer, but are overexpressed to make cells resistant to native immunity, treatment or normal senescence. Many of these genes are overexpressed in a wide array of tumors and are attractive targets for silencing. These include the genes encoding: (1) the multidrug-resistance protein, MDR, which pumps chemotherapeutic drugs out of tumors; (2) telomerase, which overcomes the chromosomal shortening that occurs with each cell division; and (3) Bcl-2, which makes cell resistant to caspase-mediated apoptosis. Other potential targets for RNAi are growth factors or growth factor receptors that have an oncogenic role in specific tumors. Examples include IL-6 for multiple myeloma and Her-2 for breast cancer. Other possible targets are viral oncogenes, such as the HPV genes E6 and E7 for cervical cancer. Synthesis of angiogenic factors by vascular endothelia or tumors could also be silenced.

In fact, the list of possible cancer targets for RNAi includes all of the genes that have been recently targeted using conventional drugs, antibodies or antisense oligonucleotides. What makes RNAi an attractive alternative is the anticipated high degree of specificity of its effect, rarely seen with small-molecule inhibitors. The possibility of hitting multiple gene targets at the same time also greatly increases the chances of success. The success of RNAi therapy for cancer, as for other indications, will depend in large part on stable and, if possible, tumor-specific delivery. The delivery methods will need to be tailor-made for each type of cancer. For example, for cervical cancer, local instillation of stabilized duplex oligonucleotides could be all that is required. However, for disseminated or hematological tumors, targeting via cell-specific receptors might work better, particularly if the target gene(s) has an essential function(s) in non-transformed cells.

Neurodegenerative disease

Another class of diseases that have defied conventional approaches, but might be amenable to RNAi, are neurodegenerative diseases caused by the overexpression of mutated genes or of proteins containing long polyglutamine stretches [65]. Targeting the expression of the mutated allele or the polyglutamine sequence could slow or prevent the onset of neuronal cell death. Interfering with the expression of apoptotic genes selectively in the nervous system could have the same effect. It is not known

whether siRNA cross the blood–brain barrier. Again, the limiting factor is elucidating the best selective delivery strategy for targeting neurons. This might require local injection into the cerebrospinal fluid of modified siRNA or of retroviruses, or lentiviruses that efficiently infect nerve cells.

Other indications

The therapeutic possibilities are endless. Almost any disease in which expression or overexpression of a native or mutated gene causes disease is a potential target, providing that low expression (~10% of wild-type) will not be toxic. Initial clinical studies will inevitably be chosen to treat ailments that afflict a large number of people, but currently have no available treatment or a suboptimal or expensive treatment.

Potential sources of toxicity

SiRNA appear to have high specificity but, like any small molecule, both sequence-specific and -nonspecific impediments to therapeutic application could arise, once the delivery issues have been solved. Targeted delivery, if possible, will reduce the likelihood of toxicity. Most side effects are also expected to be dose-dependent and could be mitigated by administering less siRNA, adjusting its half-life by chemical modification, or by using a less-potent expression system for vectored delivery. Possible side effects include unintended silencing of partially homologous genes, either by mRNA degradation or suppressing translation, or induction of global gene suppression by activating the IFN response, especially when shRNAs are expressed from viral vectors. Another potential problem caused by the therapeutic introduction of siRNA could be inhibition of the function of endogenous miRNAs by competing for the RNAi machinery.

Conclusions

RNAi provides an exciting new therapeutic tool with a wide array of potential disease targets. The specificity and effectiveness of gene silencing with RNAi promises therapies with a high therapeutic index. Devising strategies to deliver siRNA into cells in a stable and cell-specific manner is the major impediment at present. Given the rapid pace in the field, with impressive demonstrations of efficacy *in vitro* and in mice, human pilot studies to provide the proof of principle for therapeutic use are likely to be undertaken within the next few years.

References

1 Fire, A. *et al.* (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811
2 Sharp, P.A. (2001) RNA interference–2001. *Genes Dev.* 15, 485–490
3 Zamore, P.D. (2001) RNA interference: listening to the sound of silence. *Nat. Struct. Biol.* 8, 746–750
4 Hannon, G.J. (2002) RNA interference. *Nature* 418, 244–251
5 Tijsterman, M. *et al.* (2002) The genetics of RNA silencing. *Annu. Rev. Genet.* 36, 489–519
6 Elbashir, S.M. *et al.* (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411, 494–498
7 McManus, M.T. *et al.* (2002) Gene silencing in mammals by small interfering RNAs. *Nat. Rev. Genet.* 3, 737–747
8 Timmons, L. *et al.* (1998) Specific interference by ingested dsRNA. *Nature* 395, 854
9 Napoli, C. *et al.* (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans*. *Plant Cell* 2, 279–289
10 Jorgensen, R. (1990) Altered gene expression in plants due to trans interactions between homologous genes. *Trends Biotechnol.* 8, 340–344
11 Jorgensen, R.A. *et al.* (1996) Chalcone synthase cosuppression phenotypes in petunia flowers: comparison of sense vs antisense constructs and single-copy vs complex T-DNA sequences. *Plant Mol. Biol.* 31, 957–973
12 Sijen, T. *et al.* (2001) On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* 107, 465–476
13 Schiebel, W. *et al.* (1998) Isolation of an RNA-directed RNA polymerase-specific cDNA clone from tomato. *Plant Cell* 10, 2087–2101
14 Doench, J.G. *et al.* (2003) siRNAs can function as miRNAs. *Genes Dev.* 17, 438–442
15 Wassenegger, M. *et al.* (1994) RNA-directed *de novo* methylation of genomic sequences in plants. *Cell* 76, 567–576
16 Pal-Bhadra, M. *et al.* (2002) RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in *Drosophila*. *Mol. Cell* 9, 315–327
17 Pal-Bhadra, M. *et al.* (1999) Cosuppression of nonhomologous transgenes in *Drosophila* involves mutually related endogenous sequences. *Cell* 99, 35–46
18 Banerjee, D. *et al.* (2002) Control of developmental timing by small temporal RNAs: a paradigm for RNA-mediated regulation of gene expression. *Bioessays* 24, 119–129
19 Hutvagner, G. *et al.* (2002) A microRNA in a multiple-turnover RNAi enzyme complex. *Science* 297, 2056–2060
20 Hamilton, A.J. *et al.* (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 286, 950–952
21 Hamilton, A. *et al.* (2002) Two classes of short interfering RNA in RNA silencing. *EMBO J.* 21, 4671–4679
22 Zilberman, D. *et al.* (2003) ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. *Science* 299, 716–719
23 Tang, G. *et al.* (2003) A biochemical framework for RNA silencing in plants. *Genes Dev.* 17, 49–63
24 Zamore, P.D. *et al.* (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 101, 25–33
25 Parrish, S. *et al.* (2000) Functional anatomy of a dsRNA trigger: Differential requirement for the two trigger strands in RNA interference. *Mol. Cell* 6, 1077–1087
26 Yang, D. *et al.* (2000) Evidence that processed small dsRNAs may mediate sequence-specific mRNA degradation during RNAi in *Drosophila* embryos. *Curr. Biol.* 10, 1191–1200
27 Hammond, S.M. *et al.* (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* 404, 293–296
28 Elbashir, S.M. *et al.* (2001) Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO J.* 20, 6877–6888
29 Bernstein, E. *et al.* (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409, 363–366
30 Ketting, R.F. *et al.* (2001) Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. *Genes Dev.* 15, 2654–2659
31 Knight, S.W. *et al.* (2001) A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in *Caenorhabditis elegans*. *Science* 293, 2269–2271
32 Hall, I.M. *et al.* (2003) RNA interference machinery regulates chromosome dynamics during mitosis and meiosis in fission yeast. *Proc. Natl. Acad. Sci. U. S. A.* 100, 193–198
33 Hall, I.M. *et al.* (2002) Establishment and maintenance of a heterochromatin domain. *Science* 297, 2232–2237
34 Volpe, T.A. *et al.* (2002) Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* 297, 1833–1837
35 Kennerdell, J.R. *et al.* (1998) Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. *Cell* 95, 1017–1026
36 Tuschl, T. *et al.* (1999) Targeted mRNA degradation by double-stranded RNA *in vitro*. *Genes Dev.* 13, 3191–3197

37 Nykanen, A. *et al.* (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* 107, 309–321
38 Tabara, H. *et al.* (1999) The *rde-1* gene, RNA interference, and transposon silencing in *C. elegans*. *Cell* 99, 123–132
39 Hammond, S.M. *et al.* (2001) Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* 293, 1146–1150
40 Martinez, J. *et al.* (2002) Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* 110, 563–574
41 Hovanessian, A.G. (1991) Interferon-induced and double-stranded RNA-activated enzymes: a specific protein kinase and 2′,5′-oligoadenylate synthetases. *J. Interferon Res.* 11, 199–205
42 Silverman, R.H. (1994) Fascination with 2-5A-dependent RNase: a unique enzyme that functions in interferon action. *J. Interferon Res.* 14, 101–104
43 Katze, M.G. (1995) Regulation of the interferon-induced PKR: can viruses cope? *Trends Microbiol.* 3, 75–78
44 Stark, G.R. (1997) Genetic analysis of interferon and other mammalian signaling pathways. *Harvey Lect.* 93, 1–16
45 Gil, J. *et al.* (2000) Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): mechanism of action. *Apoptosis* 5, 107–114
46 Elbashir, S.M. *et al.* (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* 15, 188–200
47 Li, H. *et al.* (2002) Induction and suppression of RNA silencing by an animal virus. *Science* 296, 1319–1321
48 Paddison, P.J. *et al.* (2002) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.* 16, 948–958
49 Caplen, N.J. *et al.* (2001) Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc. Natl. Acad. Sci. U. S. A.* 98, 9742–9747
50 McManus, M.T. *et al.* (2002) Small interfering RNA-mediated gene silencing in T lymphocytes. *J. Immunol.* 169, 5754–5760
51 McManus, M.T. *et al.* (2002) Gene silencing using micro-RNA designed hairpins. *RNA* 8, 842–850
52 Paul, C.P. *et al.* (2002) Effective expression of small interfering RNA in human cells. *Nat. Biotechnol.* 20, 505–508
53 Miyagishi, M. *et al.* (2002) U6 promoter-driven siRNAs with four uridine 3′ overhangs efficiently suppress targeted gene expression in mammalian cells. *Nat. Biotechnol.* 20, 497–500
54 Sui, G. *et al.* (2002) A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc. Natl. Acad. Sci. U. S. A.* 99, 5515–5520
55 McCaffrey, A.P. *et al.* (2002) RNA interference in adult mice. *Nature* 418, 38–39
56 Lewis, D.L. *et al.* (2002) Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nat. Genet.* 32, 107–108
57 Garrus, J.E. *et al.* (2001) Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. *Cell* 107, 55–65
58 Brummelkamp, T. *et al.* (2002) Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell* 2, 243–247
59 Wilda, M. *et al.* (2002) Killing of leukemic cells with a BCR/ABL fusion gene by RNA interference (RNAi). *Oncogene* 21, 5716–5724
60 Jiang, M. *et al.* (2002) Selective silencing of viral gene expression in HPV-positive human cervical carcinoma cells treated with siRNA, a primer of RNA interference. *Oncogene* 21, 6041–6048
61 Bhoumik, A. *et al.* (2002) An ATF2-derived peptide sensitizes melanomas to apoptosis and inhibits their growth and metastasis. *J. Clin. Invest.* 110, 643–650
62 Cioca, D.P. *et al.* (2003) RNA interference is a functional pathway with therapeutic potential in human myeloid leukemia cell lines. *Cancer Gene Ther.* 10, 125–133
63 Song, E. *et al.* (2003) RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat. Med.* 9, 347–351
64 Zender, L. *et al.* (2003) Caspase 8 siRNA prevents acute liver failure in mice. *Proc. Natl. Acad. Sci. U. S. A.* 100, 7797–7802
65 Xia, H. *et al.* (2002) siRNA-mediated gene silencing *in vitro* and *in vivo*. *Nat. Biotechnol.* 20, 1006–1010
66 Bitko, V. *et al.* (2001) Phenotypic silencing of cytoplasmic genes using sequence-specific double-stranded short interfering RNA and its application in the reverse genetics of wild type negative-strand RNA viruses. *BMC Microbiol.* 1, 34
67 Lee, N.S. *et al.* (2002) Expression of small interfering RNAs targeted against HIV-1 *rev* transcripts in human cells. *Nat. Biotechnol.* 20, 500–505
68 Novina, C.D. *et al.* (2002) siRNA-directed inhibition of HIV-1 infection. *Nat. Med.* 8, 681–686
69 Jacque, J.M. *et al.* (2002) Modulation of HIV-1 replication by RNA interference. *Nature* 418, 435–438
70 Hu, W. *et al.* (2002) Inhibition of retroviral pathogenesis by RNA interference. *Curr. Biol.* 12, 1301–1311
71 Coburn, G.A. *et al.* (2002) Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. *J. Virol.* 76, 9225–9231
72 Capodici, J. *et al.* (2002) Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference. *J. Immunol.* 169, 5196–5201
73 Gitlin, L. *et al.* (2002) Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature* 418, 430–434
74 Yokota, T. *et al.* (2003) Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. *EMBO Rep.* 4, 602–608
75 Hamasaki, K. *et al.* (2003) Short interfering RNA-directed inhibition of hepatitis B virus replication. *FEBS Lett.* 543, 51–54
76 McCaffrey, A.P. *et al.* (2003) Inhibition of hepatitis B virus in mice by RNA interference. *Nat. Biotechnol.* 21, 639–644
77 Ge, Q. *et al.* (2003) RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. *Proc. Natl. Acad. Sci. U. S. A.* 100, 2718–2723
78 Song, E. *et al.* (2003) Sustained small interfering RNA-mediated human immunodeficiency type 1 inhibition in primary macrophages. *J. Virol.* 77, 7174–7181
79 Qin, X.F. *et al.* (2003) Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. *Proc. Natl. Acad. Sci. U. S. A.* 100, 183–188
80 Jackson, A.L. *et al.* (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* 21, 635–637
81 Yu, J.Y. *et al.* (2002) RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc. Natl. Acad. Sci. U. S. A.* 99, 6047–6052
82 Zeng, Y. *et al.* (2002) Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. *Mol. Cell* 9, 1327–1333
83 Rubinson, D.A. *et al.* (2003) A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat. Genet.* 33, 401–406
84 Stewart, S.A. *et al.* (2003) Lentivirus-delivered stable gene silencing by RNAi in primary cells. *RNA* 9, 493–501
85 Wang, J. *et al.* (2003) Stable and controllable RNA interference: investigating the physiological function of glutathionylated actin. *Proc. Natl. Acad. Sci. U. S. A.* 100, 5103–5106
86 Yang, P.L. *et al.* (2002) Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection. *Proc. Natl. Acad. Sci. U. S. A.* 99, 13825–13830
87 Usman, N. *et al.* (2000) Nuclease-resistant synthetic ribozymes: developing a new class of therapeutics. *J. Clin. Invest.* 106, 1197–1202
88 Amarzguioui, M. *et al.* (2003) Tolerance for mutations and chemical modifications in a siRNA. *Nucleic Acids Res.* 31, 589–595
89 Czauderna, F. *et al.* (2003) Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. *Nucleic Acids Res.* 31, 2705–2716
90 Harborth, J. *et al.* (2003) Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. *Antisense Nucleic Acid Drug Dev.* 13, 83–105
91 Sandgren, S. *et al.* (2002) Nuclear targeting of macromolecular polyanions by an HIV-tat derived peptide. Role for cell-surface proteoglycans. *J. Biol. Chem.* 277, 38877–38883
92 Simeoni, F. *et al.* (2003) Insight into the mechanism of the peptide-based gene delivery system MPG: implications for delivery of siRNA into mammalian cells. *Nucleic Acids Res.* 31, 2717–2724
93 Bertrand, J.R. *et al.* (2002) Comparison of antisense oligonucleotides and siRNAs in cell culture and *in vivo*. *Biochem. Biophys. Res. Commun.* 296, 1000–1004
94 Sorensen, D.R. *et al.* (2003) Gene silencing by systemic delivery of synthetic siRNAs in adult mice. *J. Mol. Biol.* 327, 761–766

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Small interfering RNA (siRNA) targeting *VEGF* effectively inhibits ocular neovascularization in a mouse model

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Purpose: RNA interference mediated by small interfering RNAs (siRNAs) is a powerful technology allowing the silencing of mamalian genes with great specificity and potency. The purpose of this study was to demonstrate the feasibility of RNA interference mediated by siRNA in retinal cells in vitro and in the murine retina in vivo.

Methods: siRNAs specific for enhanced green fluorescent protein (*EGFP*) and murine and human vascular endothelial growth factor (*VEGF*) were designed. In vitro studies in human cell lines entailed modulation of endogenous VEGF levels through chemically induced hypoxia. Effects of siRNA treatment on these levels were measured by ELISA. In vivo studies evaluating effects of siRNA on levels of EGFP and VEGF were performed by co-injecting recombinant viruses carrying *EGFP* or *hVEGF* cDNAs along with the appropriate siRNAs subretinally in mice. Additional studies aimed at blocking production of endogenous mVEGF were performed using laser-induced choroidal neovascularization (CNV) in mice. Effects of in vivo treatments were evaluated ophthalmoscopically. Retinal/choroidal flat mounts were evaluated after perfusion with dextran-fluorescein. Alternatively, retinas were evaluated in histological sections or VEGF levels were measured in intact eyes using ELISA.

Results: Successful delivery of siRNA to the subretinal space was confirmed by observing significantly reduced levels of EGFP in eyes treated with Ad.CMV.EGFP plus *EGFP*-directed siRNA. siRNAs directed against *hVEGF* effectively and specifically inhibit hypoxia-induced VEGF levels in human cell lines and after adenoviral induced *hVEGF* transgene expression in vivo. In addition, subretinal delivery of siRNA directed against murine *Vegf* significantly inhibited CNV after laser photocoagulation.

Conclusions: Delivery of siRNA can be used in vitro and in vivo to target specific RNAs and to reduce the levels of the specific protein product in the targeted cells. This work suggests that RNA interference has potential for application to studies of retinal biology and for the treatment of a variety of retinal diseases, including those involving abnormal blood vessel growth.

RNA interference (RNAi) is a process conserved throughout many eukaryotic organisms, in which the presence of double stranded RNA (dsRNA) in a cell results in the destruction of mRNAs whose sequences share homology to the dsRNA. This phenomenon is now being exploited as a powerful tool for reverse genetics, and shows great promise for therapeutic applications. Elbashir et al. [1] have shown that synthetic RNAs of 21 and 22 nucleotides in length are able to mediate cleavage of the target RNA. These RNAs are termed small interfering RNA (siRNA). This group has also shown that siRNAs are involved in degrading homologous RNAs in mammalian cells [1]. Recently it was demonstrated that RNA interference mediated by siRNAs can specifically target sequence from hepatitis C virus in living mice [2,3].

Technology that allows the downregulation of expression of specific genes expressed in the retina would have numerous applications ranging from improving our understanding of the basic biology of the retina to providing therapy for blinding diseases. As a first step in evaluating the potential of retinal delivery of siRNA, we selected three target genes for study: the reporter gene, enhanced green fluorescent protein (*EGFP*) and both human (h) and murine (m) vascular endothelial growth factor (*VEGF*). We designed siRNAs directed specifically against these genes and tested their abilities to mediate RNA interference in vitro in human cell lines and in vivo in mice. The ability of siRNA molecules to silence endogenous *VEGF* gene expression was also evaluated in an animal model for age-related macular degeneration (AMD): the murine laser-induced model of choroidal neovascularization (CNV) [4]. CNV, found in the "wet" form of AMD, is characterized by the growth of new blood vessels below the retina. While multiple pro-angiogenic proteins may be involved in CNV, VEGF has been shown in both clinical and bench research studies to play a critical role in the pathophysiology of this blinding condition [5-14]. *VEGF* is known to be upregulated in the laser photocoagulation model of CNV [15]. Our studies demonstrate that siRNA can be used to downregulate the expression of two different genes expressed in the retinal pigment epithelium (RPE). We also show that siRNA targeting *Vegf* can be used to decrease the extent of CNV in the laser photocoagulation model. These findings suggest that delivery of siRNA directed against *VEGF* may be a useful approach with which to treat retinal diseases with a neovascular component.

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METHODS

siRNA design: Selection of siRNAs was based on the characterization of siRNA by Elbashir et al [1,16]. An hVEGF5.siRNA targeting the sequence 5'-AAA CCU CAC CAA GGC CAG CAC-3' was selected. This siRNA consisted of an RNA duplex containing a sense strand 5'-ACC UCA CCA AGG CCA GCA CdTdT-3' and an antisense strand 5'-G UGC UGG CCU UGG UGA GGUdTdT-3'. For rescue of the CNV model an siRNA was designed that targets mouse *Vegf* mRNA at the sequence 5'-AAC GAU GAA GCC CUG GAG UGC-3'. The sense strand of this molecule, mVegf1.siRNA, was 5'-CGA UGA AGC CCU GGA GUG CdTdT-3' and the antisense strand was 5'-GCA CUC CAG GGC UUC AUC GdTdT-3'. The siRNA used to downregulate *EGFP* expression targeted the following sequence in *EGFP* mRNA: 5'-GGC UAC GUC CAG GAG CGC ACC-3'. The sense strand of this molecule, EGFP1.siRNA, was 5'-P GGC UAC GUC CAG CGC ACC-3' and the antisense strand was 5'-P U GCG CUC CUG GAC GUA GCC UU-3' (Pre-synthesized control siRNA green fluorescent protein duplex, Dharmacon Research Inc (Lafayette, CO)). The EGFP1.siRNA was also used as a control for studying the effects of hVEGF5.siRNA and mVegf1.siRNA. All synthetic RNA sequences were synthesized and purified by Dharmacon Research, Inc.

Preparation of recombinant adenoviruses: Serotype 5, E1-deleted replication defective adenovirus vectors were prepared and used as described [17,18]. Transgenes were driven by the cytomegalovirus (CMV) promoter. The viruses were prepared in human embryonic kidney '293' cells, purified by double CsCl density gradient centrifugation and stored in 10% glycerol at -70 ℃ prior to use.

siRNA transfection and hypoxia induction in vitro: Human cell lines (human embryonic kidney '293' and HeLa cells, ATCC, Manassas, VA) were seeded into 24 well plates one day prior to transfection. At the time of transfection with siRNA, the cells were about 50% confluent in 250 ∝l of complete DMEM medium. Transfections of siRNA (at 13 nM) were performed in all cells lines using the Transit TKO Transfection reagent (Mirus, Madison, WI; using guidelines provided by the manufacturer). Controls included transfection reagent lacking siRNA, and nonspecific siRNA (EGFP1.siRNA). 24 h after transfection, hypoxia was induced in the cells by the addition of desferrioxamine to a final concentration of 130 mM in each well as described [19]. 48 h post transfection the supernatant was removed from all wells and a human VEGF ELISA (R & D systems, Minneapolis, MN) was performed on the cell supernatants as described in the Quantikine human VEGF ELISA protocol. ELISA results were read on a Wallac Victor2 ELISA plate reader (Perkin Elmer Life Sciences, Inc., Boston, MA). Experiments were repeated four times using 293 cells and three times using HeLa cells. The Student's t-test was used for statistical analysis using the combined data from each of the replicates.

In vivo studies and tissue analysis: Animal experiments were performed in accordance with institutional guidelines for the care and use of animals in research. Both adenovirus and siRNA were delivered subretinally to five adult C57Bl/6 mice (Jackson Labs, Bar Harbor, ME) as described previously [17]. The mixture injected contained about $1x10^8$ particles of Ad.CMV.EGFP, generously provided by Dr. J. Wilson (Dept. of Medical Genetics, University of Pennsylvania), and 20 picomoles of EGFP1.siRNA conjugated with transit TKO reagent (Mirus). As positive control, contralateral eyes received a mixture containing the same amount of Ad.CMV.EGFP and 20 picomoles of hVEGF5.siRNA conjugated with transit TKO reagent (Mirus). Production of EGFP was detected by ophthalmoscopy 48-60 h after injection as described [20]. Animals were sacrificed and the eyes were enucleated and fixed in 4% paraformaldehyde. Eyes were prepared as either flat mounts (4 animals/8 eyes) or as 10 ∝m cryosections for fluorescent microscopy (1 animal/2 eyes). Microscopic examinations were performed with a Zeiss fluorescent dissecting microscope and with a Leica DMR microscope (Wetzlar, Germany) equipped with epifluorescence illumination. Images were captured using a Hamamatsu CCD camera (Hamamatsu Photonics, Bridgewater, NJ) using OpenLab 2.2 software (Improvision, Boston, MA).

Inhibition of human VEGF in vivo: Ad.CMV.hVEGF, generously provided by Dr. M. Herlyn (Molecular and Cellular Oncogenesis, University of Pennsylvania), was injected subretinally and bilaterally in eyes of five C57Bl/6 mice. One eye of each animal was co-injected with mVegf1.siRNA and contralateral eyes were co-injected with EGFP1.siRNA as control. Two days later, eyes were snap frozen in liquid N_2 following enucleation. All eyes were homogenized in lysis buffer (Roche, Basel, Switzerland) and total protein was measured using a Bradford assay. The samples were all normalized for total protein prior to assaying for human VEGF by ELISA. The ELISA was performed according to the manufacturers recommendations (R & D systems).

Laser induced CNV model studies: Adult (8-15 week old) female C57Bl/6 mice (n=30) were anesthetized with avertin (2,2,2-tribromoethanol) and pupils were dilated with 1% tropicamide. Laser photocoagulation was performed bilaterally using a diode laser photocoagulator (IRIS Medical, Mountain View, CA) and slit lamp system with a cover slip as a contact lens. Laser photocoagulation (140 mW, 75 ∝m spot size, 0.1 s duration) was applied to the 8 and 10 o'clock positions in the right eye and 2 and 4 o'clock positions in the left eye, 2 to 3 disk diameters from the optic nerve. Since the rupture of Bruch's membrane is necessary to create significant CNV [4], bubble formation at the time of photocoagulation was used as an indication of the rupture of Bruch's membrane. Laser burns that did not induce a rupture in Bruch's membrane were excluded from the study. Lesions in which two laser spots became confluent were also excluded from the study.

Approximately 36 h after laser treatment, siRNA was delivered to both eyes by subretinal injection. A mixture containing about $1x10^8$ particles of Ad.CMV.LacZ, generously provided by Dr. J. Wilson, and 20 picomoles of mVegf1.siRNA conjugated with transit TKO reagent (Mirus) was injected. As control, contralateral eyes received a mixture of the same

molecules except that mVegf1.siRNA was replaced with EGFP1.siRNA. Two weeks after laser photocoagulation, animals were perfused with high molecular weight dextran-fluorescein (Molecular Probes, Eugene, OR) to label the retinal/choroidal vasculature and eyes were harvested. The area of CNV was measured in choroidal flat mount preparations by a masked individual using modifications of methods described previously [4,21]. These modifications are as follows: Microscopic examinations were performed with a Leica DMR microscope (Wetzlar, Germany) equipped with epifluorescence illumination. Lesions in the dextran-fluorescein-perfused flat mount preparations were identified as circular fluorescent (fluorescein positive) areas corresponding with the area previously exposed to the laser light. Images of the lesions were captured using a black and white Hamamatsu CCD camera (Hamamatsu Photonics, Bridgewater, NJ) coupled to a Apple Macintosh G4 computer (Cupertino, CA) equipped with OpenLab 2.2 software. Images for calibration were obtained from a slide with a grating of known size. The hyperfluorescent fluorescein-dextran labeled blood vessels within the area of the laser burn were selected as a "region of interest" (ROI) using Openlab software and this software was used to calculate the area ($\propto m^2$) occupied by the white pixels in the ROIs. The ROIs were selected after collecting the images under identical integration settings. They were selected by using the Openlab "magic wand" tool to identify pixels in the laser burn site at a range of 2000-4090 intensity units. (The intensity units are defined within the Openlab software. The units selected represented levels measured in normal fluorescein-perfused vasculature. For reference, the intensity of background, non-fluorescent, areas was <450 intensity units.) The ROIs were generally well-circumscribed by a region lacking fluorescence. After measuring the areas of CNV, images were colorized in Openlab by applying an intensity ramp at 515 nm (the wavelength at which the image data were captured) using the "Apply wavelength" function. This intensity ramp was applied to all of the pixels in the image and made the whitest pixels the brightest green color. The images were then exported to Adobe Photoshop software for presentation purposes. Situations in which there was no evidence of a laser burn after bright field analysis of choroidal flatmounts were excluded. Statistical analysis of the results was performed using a one-tailed distribution, two sample unequal variance Student's t-test.

RESULTS

Hypoxia-induced upregulation of human VEGF is halted by siRNA application in vitro: The ability of hVEGF5.siRNA to target human *VEGF* mRNA was tested in vitro in a system whereby exposure to desferrioxamine results in the induction of hypoxia-signaling events. That, in turn, induces *VEGF* expression [19].

Prior to hypoxia induction, two different human cell lines (embryonic kidney ["293"] cells and ovarian carcinoma [HeLa] cells) were transfected with hVEGF5.siRNA. As control, additional cells were transfected with an siRNA (EGFP1.siRNA) designed to target the reporter gene, *EGFP*, or with buffer alone. *VEGF* upregulation occurs due to a desferrioxamine-mediated induction of the HIF-1 hypoxic signaling pathway within 24 h [19]. To upregulate *VEGF* via this method, cells were exposed to desferrioxamine 24 h post-transfection. A human VEGF ELISA was performed on cell supernatants 24 h after desferrioxamine withdrawal in order to measure the effects of siRNA treatment on hVEGF levels. The hypoxia-mediated increase in hVEGF protein was reduced significantly in the presence of hVEGF5.siRNA (Figure 1). Exposure to the non-specific siRNA (EGFP1.siRNA) or to buffer lacking siRNA had minimal effect on hVEGF levels. EGFP1.siRNA

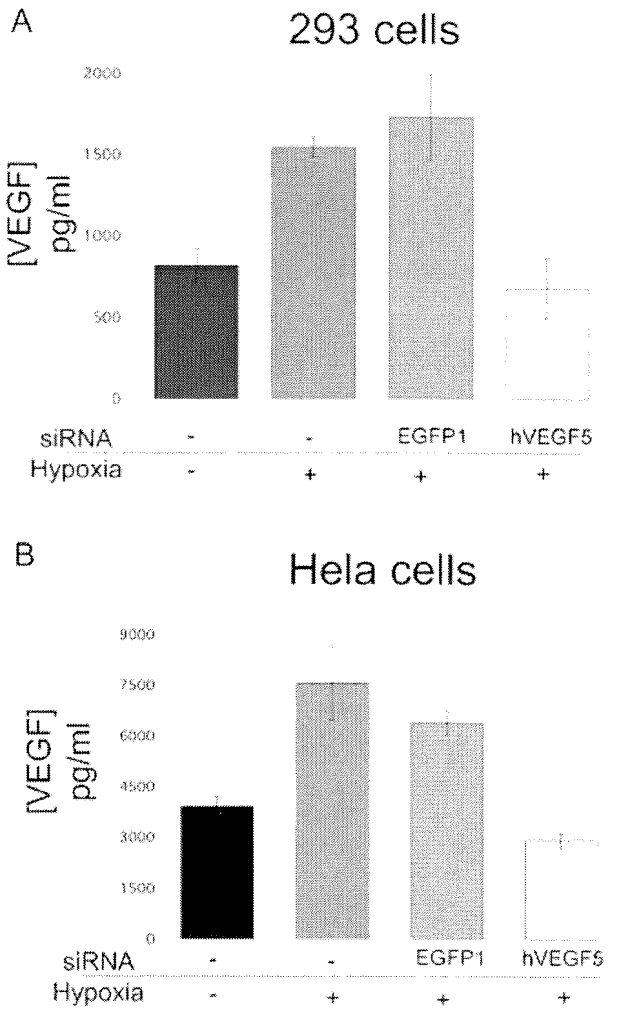


Figure 1. RNA interference disrupts desferrioxamine induced up-regulation of VEGF in vitro. The hVEGF5.siRNA or control reagents were transfected into 293 cells (**A**) and Hela cells (**B**) prior to inducing hypoxia with desferrioxamine. VEGF levels were measured in cell supernatants by ELISA 24 h after hypoxia induction. Hypoxia induced VEGF expression. In both 293 cells and HeLa cells, exposure to hVEGF1.siRNA reduced the amount of VEGF produced after hypoxia ($p<0.01$ for both cell types). There was no significant difference in the amount of VEGF produced by hypoxic cells when control reagents (EGFP1.siRNA) or no siRNA were delivered. The error bars represent the standard errors of the mean of 4 or 3 replicates, respectively, for the 293 and HeLa cells.

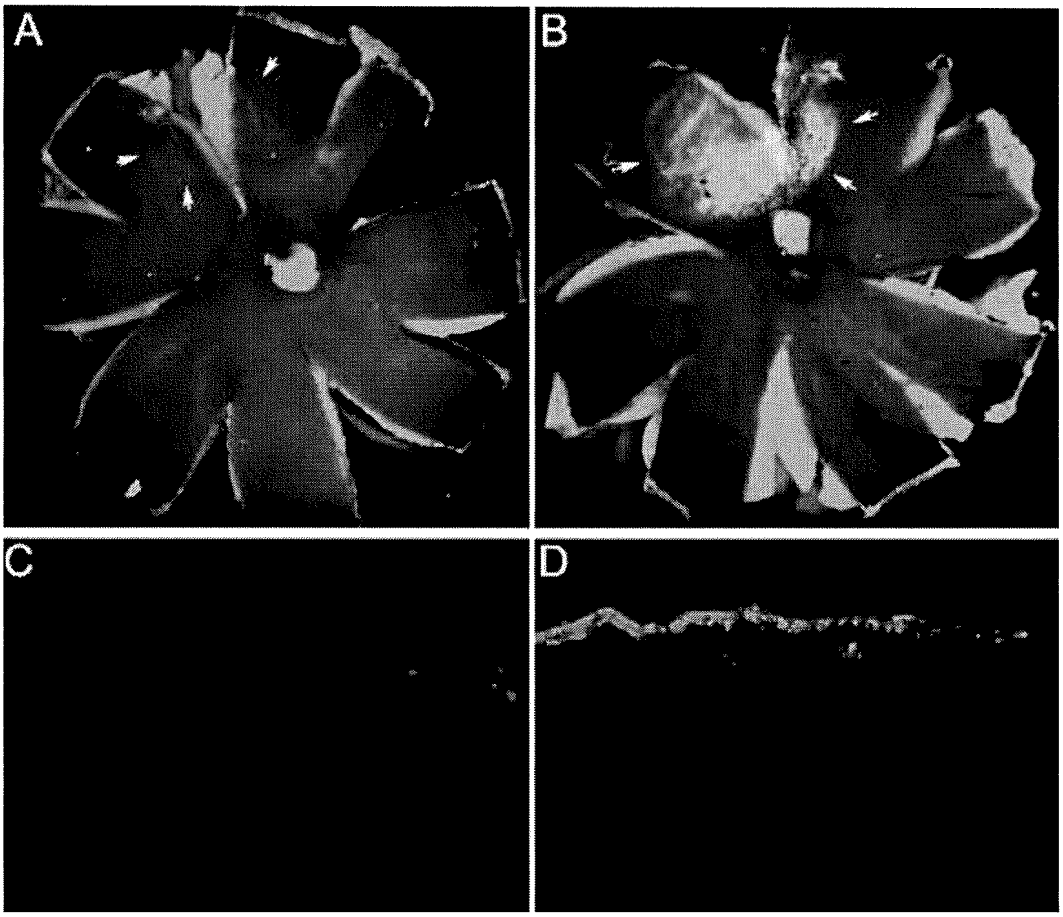


Figure 2. Effect of EGFP1.siRNA on *EGFP* expression in RPE cells in vivo. Eyes of mice were injected subretinally with Ad.CMV.EGFP in the presence of either EGFP1.siRNA (**A**,**C**) or hVEGF5.siRNA (**B**,**D**). Levels of EGFP were low in the injected portion of EGFP1.siRNA-treated eyes on inspection of flat mount preparations (**A**) and cryosections (**C**). No EGFP was detectable in the uninjected portions of these retinas. In comparison, high levels of EGFP were present in the region of injection in flat mounts (**B**) or cryosections (**D**) of eyes exposed to the control hVEGF5.siRNA. Arrows point to boundaries of injection blebs. Sections within the regions demarcated by the arrows in panels **A** and **B** are shown in panels **C** and **D**, respectively.

had no significant inhibitory effect on the concentration of hVEGF after exposure to hypoxic conditions in both 293 cells (Figure 1A; p=0.22) and HeLa cells (Figure 1B; p=0.15). There was no significant difference in hVEGF levels comparing normoxic and hypoxic, hVEGF5.siRNA-treated 293 cells (p=0.17). Human VEGF levels were significantly lower in the hypoxia plus hVEGF5.siRNA-treated cells than in cells treated with normoxia alone (p<0.01).

The ability to down-regulate VEGF production with an siRNA in vitro prompted us to explore the in vivo application of siRNA in the retina.

In vivo delivery of siRNAs to murine retinal pigment epithelial cells: Retinal pigment epithelial cells are thought to be the cells that, through abnormal VEGF production, initiate the process that leads to pathologic neovascularization (i.e. CNV) in AMD [12-14]. To determine whether siRNAs can be effectively delivered to the RPE, we expressed *EGFP* in those cells in vivo using a recombinant adenovirus, a virus that targets these cells efficiently and nearly exclusively after subretinal injection in adult mice. Recombinant adenovirus also results in a rapid onset of transgene expression [17,22]. In this system, we tested the possibility that EGFP1.siRNA would reduce levels of EGFP protein. Five mice received bilateral subretinal injections with Ad.CMV.EGFP, an E1/E3-deleted recombinant adenovirus delivering the *EGFP* cDNA driven by the CMV promoter. In the right eyes EGFP1.siRNA was also injected and in the contralateral eyes an siRNA unrelated to *EGFP* (hVEGF5.siRNA) was injected as a non-specific control. Ophthalmoscopy was performed 48 h post injection, the time at which adenovirus-mediated transgene expression in this system is maximal [17]. Ophthalmoscopy in-

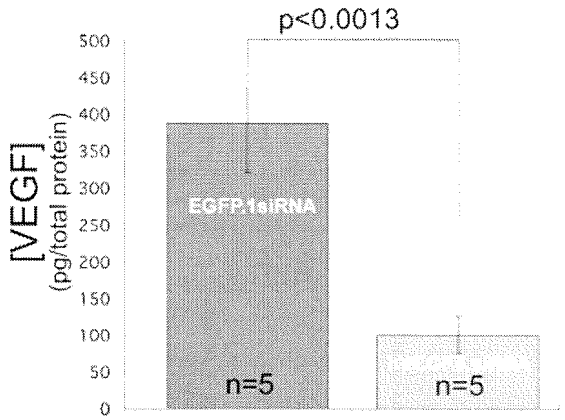
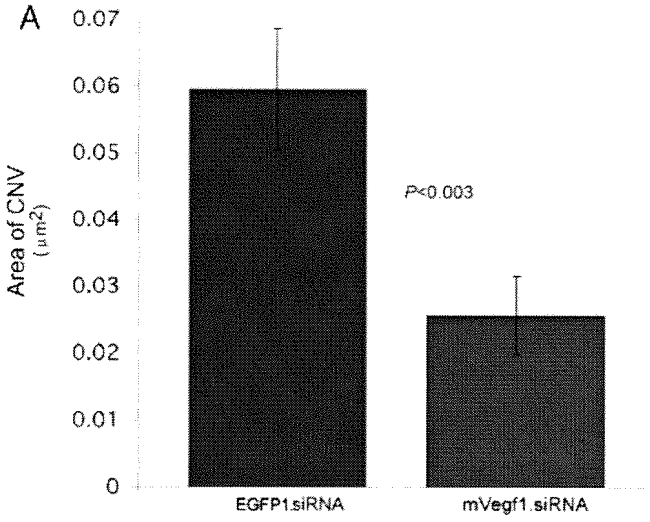


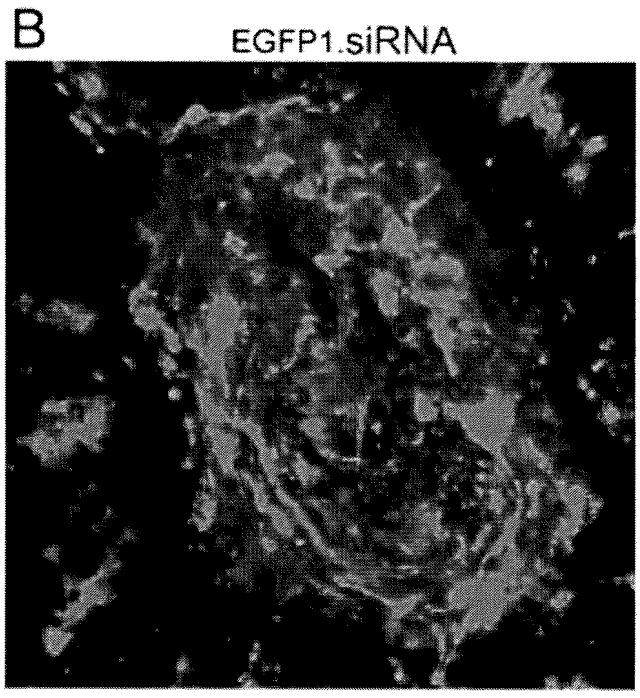
Figure 3. RNA interference significantly diminishes levels of hVEGF in vivo. Ad.CMV.hVEGF was delivered by subretinal injection bilaterally in 5 animals in the presence of either hVEGF5.siRNA or, in contralateral eyes, EGFP1.siRNA as control. Animals were sacrificed 48 h post-injection, and the eyes were removed and processed for VEGF ELISA. Bars represent the means, the error bars represent the standard errors of the mean. Injection of hVEGF5.siRNA results in significant reduction in levels of hVEGF compared to injection of EGFP1.siRNA (p<0.0013).

dicated that levels of EGFP were significantly lower in 4 of the 5 eyes co-injected with EGFP1.siRNA compared to the 5 eyes co-injected with hVEGF5.siRNA (not shown). The mice were sacrificed 48-60 h post-injection, and the eyes were harvested and evaluated for presence and amount of EGFP by inspection of retinal flat mounts and histological preparations. Figure 2A,B is representative of the flat mount results. There were only low levels of EGFP-specific fluorescence in whole mount preparations of the eyes that had received EGFP1.siRNA (Figure 2A) whereas EGFP was intensely fluorescent in retinas co-injected with hVEGF5.siRNA (Figure 2B). Uninjected portions of the eyes showed only background levels of fluorescence. Histological analyses revealed faint levels of EGFP in occasional cells of the injected portions of the retinas co-injected with EGFP1.siRNA (Figure 2C) but high levels of EGFP in the analogous regions of the contralateral eyes co-injected with hVEGF5.siRNA (Figure 2D).

Adenoviral expression of human VEGF *silenced by siRNA in murine retina in vivo:* Having demonstrated delivery of siRNA to RPE cells and significant diminution in levels of a marker protein, we sought to inhibit expression of a biologically relevant molecule in the retina in vivo. *VEGF* is an ideal candidate as this molecule is known to play a significant role in retinal neovascular disease [8-11]. Ad.CMV.hVEGF was used to deliver *hVEGF* as this virus can upregulate pharmacological levels of hVEGF and is capable of producing CNV when injected in the subretinal space [23,24].

One eye of each of 5 animals was co-injected with Ad.CMV.hVEGF and hVEGF5.siRNA while contralateral eyes were co-injected with Ad.CMV.hVEGF and EGFP1.siRNA as control. The animals were sacrificed 60 h post-injection and the eyes were processed for a human VEGF ELISA. There was a significant attenuation of VEGF levels in eyes that had received hVEGF5.siRNA as compared to the control





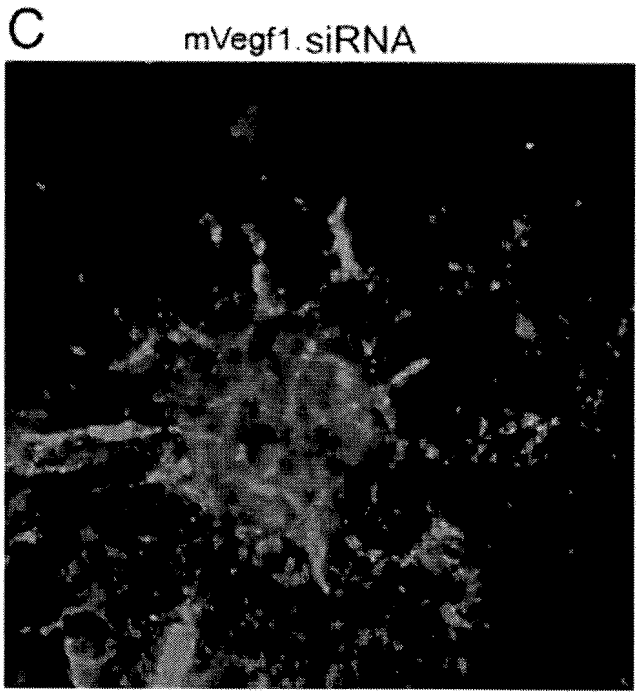


Figure 4. The extent of CNV is significantly reduced after subretinal delivery of mVegf1.siRNA. Thirty-six hours following laser photocoagulation, mVegf1.siRNA was delivered subretinally in one eye of each of 30 mice. Contralateral eyes were injected with a control (EGFP1) siRNA. Animals were perfused with dextran-fluorescein and the areas of CNV were measured in choroidal flat mounts 14 days after laser treatment. There is a significant difference ($p<0.003$) in mean areas of CNV between eyes injected with mVegf1.siRNA versus EGFP1.siRNA (panel **A**). Representative areas of CNV in eyes of a dextran-fluorescein-perfused animal that had received EGFP1.siRNA in one eye but mVegf1.siRNA in the other are shown in the colorized panels **B** and **C**, respectively. The CNV lesions were generally well-circumscribed by a region lacking fluorescence (as in panel **B**). CNV was identified by observing dextran-fluorescein-filled blood vessels on the choroidal/retinal interface, which are normally absent.

EGFP1.siRNA (Figure 3). The data demonstrate that the *hVEGF* siRNA is effective in decreasing levels of hVEGF in murine RPE cells in vivo.

Inhibition of choroidal neovascularization using siRNA directed at murine Vegf: To determine whether siRNA can inhibit CNV in an animal model, we tested the effects of delivery of an siRNA directed against murine *Vegf* (mVegf1.siRNA) in a laser induced model of CNV. We delivered mVegf1.siRNA by subretinal injection to the RPE of mice 36 h after laser treatment. The areas of mVegf1.siRNA injection encompassed all of the laser spots as well as untreated retina adjacent to these spots. Contralateral eyes received siRNA targeting *EGFP* (EGFP1.siRNA) as control. The mice were perfused with dextran-fluorescein 14 days after the laser treatment, the time of maximal neovascularization, and the areas of neovascularization were measured using digital image capture around the burn spots. The majority of lesions (75% for the EGFP1.siRNA-treated eyes and 80% for the Vegf1.siRNA-treated eyes) met the criteria for analysis (see Methods). The locations of these neovascular areas exactly coincided with the sites initially exposed to laser. Neovascularization was not observed in portions of the retina that had not been exposed to laser. The areas of neovascularization in animals that received mVegf1.siRNA were, on average, one quarter of the area of the control-treated spots (Figure 4, $p<0.003$). These data show that siRNA targeting *mVegf* is capable of inhibiting CNV in the laser photocoagulation model.

DISCUSSION

The data presented here represent the application of a technology to inhibit expression of genes in the retina, specifically to inhibit the expression of *VEGF*. To our knowledge, the data presented here describe the first successful application of siRNA to the retina. siRNA was used to significantly decrease levels of exogenous expression of transgenes both in vitro and in vivo. Results shown here from in vitro studies using siRNA directed against *VEGF* revealed a significant reduction in the amount of VEGF protein produced under hypoxic conditions. Even more impressive due to the high levels of protein produced after delivery of recombinant adenovirus, siRNA directed against *VEGF* significantly reduced VEGF protein levels after delivery of Ad.CMV.hVEGF in vivo. Finally, a test for relevance of *VEGF*-directed siRNA towards human retinal disease comes from studying its effect in an in vivo model of CNV. In accord with the in vitro and the in vivo Ad.CMV.hVEGF data, application of a *Vegf*-directed siRNA significantly reduced the extent of neovascularization in the murine laser photocoagulation model of CNV. The results provide an encouraging first step in application of siRNA technology to the retina.

As with any new technology, the data from the siRNA studies presented here invite many questions. For example: how can this technology be applied to other (non RPE) retinal cell types? While these data demonstrate delivery of functional siRNA to the RPE they do not explore delivery of the molecules to other retinal cells. Subretinal delivery of adenovirus efficiently targets the RPE and occasional Müller cells, but not other layers of the adult retina [17]. Because the reporter protein was only expressed in the RPE we cannot currently assess the delivery of siRNA to other retinal cell types. We are currently employing other assays to address efficient delivery to the photoreceptors, Müller cells and ganglion cells. What is the stability of the effect? Is there any toxicity induced by the delivery or the composition of the siRNAs? Although there was no qualitative evidence of acute toxicity in the small number of samples we evaluated, it will be important to formally evaluate both acute and chronic toxicity of the treatment/siRNA in a larger number of eyes. In the case of laser photocoagulation/CNV, how efficacious is the effect of VEGF.siRNA when it is administered at different stages of the disease? Will siRNA-mediated therapy be effective in other animal models of retinal neovascularization? VEGF siRNA-mediated rescue of additional models of ocular neovascularization such as the murine retinopathy of prematurity model [25] and models in larger animals are currently being studied in pursuit of answers to these questions.

The stability of the siRNA-induced interference effect on retinal cells is unknown at present. Experiments in progress aim to characterize the timecourse and magnitude of the effect after siRNA transfection. If this effect is found to be short-lived, there are other modifications that could be used to achieve a more sustained effect. For example, the combination of siRNA technology with recombinant viral vector techniques is a particularly promising avenue. Virus-mediated delivery of siRNA would allow delivery of therapeutic siRNA molecules that are replenished over time. Regardless of the mode of delivery, the potential of siRNA for contributing to a diverse set of applications is exciting. The possibilities range from using this technology to define developmental and physiological processes in the retina to testing approaches that might result in therapy for a diverse set of ocular diseases, including those involving ocular neovascularization.

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REFERENCES

1. Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev 2001; 15:188-200.
2. McCaffrey AP, Meuse L, Pham TT, Conklin DS, Hannon GJ, Kay MA. RNA interference in adult mice. Nature 2002; 418:38-9.
3. Song E, Lee SK, Wang J, Ince N, Ouyang N, Min J, Chen J, Shankar P, Lieberman J. RNA interference targeting Fas protects mice from fulminant hepatitis. Nat Med 2003; 9:347-51.
4. Tobe T, Ortega S, Luna JD, Ozaki H, Okamoto N, Derevjanik NL, Vinores SA, Basilico C, Campochiaro PA. Targeted disruption

of the FGF2 gene does not prevent choroidal neovascularization in a murine model. Am J Pathol 1998; 153:1641-6.
5. Lu M, Adamis AP. Vascular endothelial growth factor gene regulation and action in diabetic retinopathy. Ophthalmol Clin North Am 2002; 15:69-79.
6. Campochiaro PA. Retinal and choroidal neovascularization. J Cell Physiol 2000; 184:301-10.
7. Kwak N, Okamoto N, Wood JM, Campochiaro PA. VEGF is major stimulator in model of choroidal neovascularization. Invest Ophthalmol Vis Sci 2000; 41:3158-64.
8. Aiello LP, Avery RL, Arrigg PG, Keyt BA, Jampel HD, Shah ST, Pasquale LR, Thieme H, Iwamoto MA, Park JE, et al. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N Engl J Med 1994; 331:1480-7.
9. Krzystolik MG, Afshari MA, Adamis AP, Gaudreault J, Gragoudas ES, Michaud NA, Li W, Connolly E, O'Neill CA, Miller JW. Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment. Arch Ophthalmol 2002; 120:338-46.
10. Tolentino MJ, McLeod DS, Taomoto M, Otsuji T, Adamis AP, Lutty GA. Pathologic features of vascular endothelial growth factor-induced retinopathy in the nonhuman primate. Am J Ophthalmol 2002; 133:373-85.
11. Adamis AP, Shima DT, Tolentino MJ, Gragoudas ES, Ferrara N, Folkman J, D'Amore PA, Miller JW. Inhibition of vascular endothelial growth factor prevents retinal ischemia-associated iris neovascularization in a nonhuman primate. Arch Ophthalmol 1996; 114:66-71.
12. Grossniklaus HE, Ling JX, Wallace TM, Dithmar S, Lawson DH, Cohen C, Elner VM, Elner SG, Sternberg P Jr. Macrophage and retinal pigment epithelium expression of angiogenic cytokines in choroidal neovascularization. Mol Vis 2002; 8:119-26 .
13. Hofman P, Blaauwgeers HG, Tolentino MJ, Adamis AP, Nunes Cardozo BJ, Vrensen GF, Schlingemann RO. VEGF-A induced hyperpermeability of blood-retinal barrier endothelium in vivo is predominantly associated with pinocytotic vesicular transport and not with formation of fenestrations. Vascular endothelial growth factor-A. Curr Eye Res 2000; 21:637-45.
14. Lopez PF, Sippy BD, Lambert HM, Thach AB, Hinton DR. Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related choroidal neovascular membranes. Invest Ophthalmol Vis Sci 1996; 37:855-68.
15. Shen WY, Yu MJ, Barry CJ, Constable IJ, Rakoczy PE. Expression of cell adhesion molecules and vascular endothelial growth factor in experimental choroidal neovascularisation in the rat. Br J Ophthalmol 1998; 82:1063-71.
16. Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, Tuschl T. Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. EMBO J 2001; 20:6877-88.
17. Bennett J, Wilson J, Sun D, Forbes B, Maguire A. Adenovirus vector-mediated in vivo gene transfer into adult murine retina. Invest Ophthalmol Vis Sci 1994; 35:2535-42.
18. Gruss CJ, Satyamoorthy K, Berking C, Lininger J, Nesbit M, Schaider H, Liu ZJ, Oka M, Hsu MY, Shirakawa T, Li G, Bogenrieder T, Carmeliet P, El-Deiry WS, Eck SL, Rao JS, Baker AH, Bennet JT, Crombleholme TM, Velazquez O, Karmacharya J, Margolis DJ, Wilson JM, Detmar M, Skobe M, Robbins PD, Buck C, Herlyn M. Stroma formation and angiogenesis by overexpression of growth factors, cytokines, and proteolytic enzymes in human skin grafted to SCID mice. J Invest Dermatol 2003; 120:683-92.
19. Jiang BH, Zheng JZ, Leung SW, Roe R, Semenza GL. Transactivation and inhibitory domains of hypoxia-inducible factor 1alpha. Modulation of transcriptional activity by oxygen tension. J Biol Chem 1997; 272:19253-60.
20. Bennett J, Duan D, Engelhardt JF, Maguire AM. Real-time, noninvasive in vivo assessment of adeno-associated virus-mediated retinal transduction. Invest Ophthalmol Vis Sci 1997; 38:2857-63.
21. Edelman JL, Castro MR. Quantitative image analysis of laser-induced choroidal neovascularization in rat. Exp Eye Res 2000; 71:523-33.
22. Li T, Adamian M, Roof DJ, Berson EL, Dryja TP, Roessler BJ, Davidson BL. In vivo transfer of a reporter gene to the retina mediated by an adenoviral vector. Invest Ophthalmol Vis Sci 1994; 35:2543-9.
23. Spilsbury K, Garrett KL, Shen WY, Constable IJ, Rakoczy PE. Overexpression of vascular endothelial growth factor (VEGF) in the retinal pigment epithelium leads to the development of choroidal neovascularization [published erratum appears in Am J Pathol 2000; 157:1413]. Am J Pathol 2000; 157:135-44.
24. Baffi J, Byrnes G, Chan CC, Csaky KG. Choroidal neovascularization in the rat induced by adenovirus mediated expression of vascular endothelial growth factor. Invest Ophthalmol Vis Sci 2000; 41:3582-9.
25. Smith LE, Wesolowski E, McLellan A, Kostyk SK, D'Amato R, Sullivan R, D'Amore PA. Oxygen-induced retinopathy in the mouse. Invest Ophthalmol Vis Sci 1994; 35:101-11.

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Inhibition of GM-CSF Receptor Function by Stable RNA Interference in a NOD/SCID Mouse Hematopoietic Stem Cell Transplantation Model

MICHAELA SCHERR, KARIN BATTMER, IRIS DALLMANN, ARNOLD GANSER, and MATTHIAS EDER

ABSTRACT

RNA interference (RNAi) describes a highly conserved mechanism of sequence-specific posttranscriptional gene silencing triggered by double-stranded RNA (dsRNA). Whereas RNAi is applied to study gene function in different organisms and in variant cell types, little is known about RNAi in human hematopoietic stem and progenitor cells and their myeloid progeny. To address this issue, short hairpin RNAs (shRNA) were designed to target the common β-chain of the human receptors for granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and IL-5 (β-GMR). These receptors regulate proliferation, survival, differentiation, and functional activity of hematopoietic cells. In addition to markedly inhibiting mRNA and protein expression, anti-β-GMR shRNAs were also found to inhibit receptor function in a cell culture model. Furthermore, lentiviral gene transfer of shRNA expression cassettes into primary normal $CD34^+$ cells selectively inhibited colony formation of transduced progenitors when stimulated with GM-CSF/IL-3 but not when stimulated with cytokines that do not signal via β-GMR. Finally, anti-β-GMR shRNAs had no detectable effect on engraftment or lineage composition of lentivirally transduced human $CD34^+$ cells transplanted into NOD/SCID mice. However, the growth defect of transduced colony-forming cells under stimulation with GM-CSF/IL-3 remains unchanged in bone marrow cells harvested from individual NOD/SCID mice 6 weeks after transplantation. These data indicate that lentiviral gene transfer of shRNA expression cassettes may be used to induce long-term RNAi in human hematopoietic stem and progenitor cells for functional genetics and potential therapeutic intervention.

INTRODUCTION

RNA INTERFERENCE (RNAi) DESCRIBES a highly conserved regulatory mechanism of sequence-specific RNA depletion that is initiated by homologous double-stranded RNA (dsRNA). RNAi-mediated gene silencing can be triggered by variant forms of dsRNA, such as small hairpin RNA (shRNA) or small interfering RNA (siRNA), and it is now being used as a tool for functional genomics in multiple organisms (Fire et al., 1998; Ngo et al., 1998; Gonczy et al., 2000; Fraser et al., 2000; Catalanotto et al., 2000; Wianny and Zernicka-Goetz, 2000; Elbashir et al., 2001; Pal-Bhadra et al., 2002; Ashrafi et al., 2003; Kamath et al., 2003; Roignant et al., 2003). In mammalian cells, including human, RNAi induces transient gene silencing after a single siRNA application, whereas stable intracellular transcription of RNAi triggers can induce long-term inhibition of gene expression (Devroe and Silver, 2002; Barton and Medzhitov, 2002; Brummelkamp et al., 2002; Abbas-Terki et al., 2002; Xia et al., 2002; Rubinson et al., 2003; Kunath et al., 2003; Stewart et al., 2003; Scherr et al., 2003a).

Hematopoiesis is a hierarchically ordered system with a pool of self-renewing and pluripotent hematopoietic stem cells (HSC) generating committed progenitors that finally differentiate into mature blood cells of all hematopoietic

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lineages. The proliferation and differentiation of hematopoietic cells are partly regulated by hematopoietic cytokines, such as interleukin-3 (IL-3), erythropoietin (EPO), stem cell factor (SCF), thrombopoietin (TPO), granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte CSF (G-CSF) (for review, see Metcalf, 1989). Hematopoietic cytokines initiate their effects by binding to specific cell surface receptors. On ligand binding, these receptors become activated and subsequently transducer specific intracellular signals involving induction of tyrosine kinase activity (for review, see Ihle, 1995).

Until now, RNAi in hematopoiesis has been studied mostly in lymphatic cell lines or primary T lymphocytes in the context of HIV infection (Martinez et al., 2002; McManus et al., 2002; Capodici et al., 2002; Coburn and Cullen, 2002; Jacque et al., 2002; Banerjea et al., 2003; Qin et al., 2003). In contrast, little is known about RNAi in $CD34^+$ stem and progenitor cells and their myeloid progeny. Our group recently reported first results on specific RNAi against the common β-chain of the receptors for GM-CSF, IL-3, and IL-5 (referred to as β-GMR) in primary $CD34^+$ colony-forming cells. The human receptors for GM-CSF, IL-3, and IL-5 have unique α-chains required for ligand binding but share a common β-chain (β-GMR) essential for intracellular signal transduction. Using the β-GMR model that allows specific analysis of receptor function by the selective choice of cytokine stimulation for *in vitro* cultures, we demonstrated stable RNAi in $CD34^+$ colony-forming progenitor cells on lentiviral gene transfer of shRNA expression cassettes (Scherr et al., 2003a). In contrast to colony-forming cells, which represent late hematopoietic progenitors, there are no reports to date on stable RNAi in human HSC. For example, it is not known to what extent primary human HSC can be genetically modified to inhibit endogenous gene expression by stable RNAi. Additionally, the impact of expression of sh/siRNA on engraftment capability and differentiation potential of HSC has not been reported.

To address these questions, we expressed selected anti β-GMR shRNAs from lentiviral H1 expression cassettes, and we demonstrate the inhibition of mRNA and protein expression as well as RNAi-mediated inhibition of GMR signal transduction in a cell culture model. We performed xenotransplantation of primary $CD34^+$ cells into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice and used NOD/SCID repopulating cells (SRC) as a surrogate to analyze human HSC (Dick et al., 1997). We show specific and nearly identical inhibition of β-GMR function in colony-forming cells both before and after transplantation of $CD34^+$ cells into NOD/SCID mice without detectable effects of multilineage engraftment of SRCs. These data demonstrate that stable RNAi can be induced in human HSC by lentiviral gene transfer and can mediate long-term and functional gene silencing of an endogenous hematopoiesis-specific gene.

MATERIALS AND METHODS

shRNA synthesis and construction of H1-shRNA expression cassettes

Five different DNA oligonucleotides corresponding to positions 1466–1484, 1495–1513, 1775–1793, 2095–2113, and 2194–2212 of the sequence of the common β-chain of the human receptors for GM-CSF, IL-3, and IL-5 were chemically synthesized, including overhang sequences from a 5′ BglII and a 3′ SalI restriction site for cloning purposes (BioSpring, Frankfurt, Germany). The numbering of β-GMR nucleotides refers to Hayashida et al. (1990) (GeneBank accession No. M59941). The oligonucleotide sequences were as follows: FP1466, 5′-GATCCCCCCCCCAGCAAGAGCCAC*CTG*TTCAAGAGA*CAGGTGGCTCTTGCTGGGG*-TTTTTTGGAAG-3′; RP1466, 5′-TCGACTTCCAAA-AAA*CCCCAGCAAGAGCCACCTG*TCTCTTGAA-*CAGGTGGCTCT*TGCTGGGGGGG-3′; FP1495, 5′-GA-TCCCCC*GGGAGCGCAGAGCTTTGG*TTCAAGAGA*CCAAAGCTCTGCGCTCCCG*TTTTTTGGAAG-3′; RP1495, 5′-TCGACTTCCAAAAAA*CGGGAGCGCAG-AGCTTTGG*TCTCTTGAA*CCAAAGCTCTGCGCTCC-CG*GGG-3′; FP1775, 5′-GATCCCCC*AGGCTTCCAG-CTTTGAC*TTCAAGAGA*AGTCAAAGCTGGAAGCCT G*TTTTTTGGAAG-3′; RP1775, 5′-TCGACTTCCAA-AAAA*CAGGCTTCCAGCTTTGACT*TCTCTTGAA*AGT CAAAGCTGGAAGCCTG*GGG-3′; FP2095β, 5′-GATC-CCC*GGACAGCCCTGTGGCTATA*TTCAAGAGA*TA-TAGCCACAGGGCTGTCC*TTTTTTGGAAG-3′; RP2095β, 5′-TCGACTTCCAAAAAA*GGACAGCCCT-GTGGCTATA*TCTCTTGAA*TATAGCCACAGGGCT-GTCC*GGG-3′; FP2194, 5′-GATCCCCC*TCAGGGGC-CTCGTCTGTC*TTTCAAGAGA*GACAGACGAGGCCC CTGAG*TTTTTTGGAAG-3′; RP2194, 5′-TCGACTTC-CAAAAAA*CTCAGGGGCCTCGTCTGTC*TCTCTT-GAA*GACAGACGAGGCCCCTGAG*GGG-3′. The non-complementary 9-nt loop sequences are underlined, and each sense oligonucleotide harbors a stretch of T as a polymerase III (pol III) transcription termination signal. Corresponding oligonucleotides were annealed and inserted 3′ of the H1-RNA promoter into the BglII/SalI-digested pBS-H1 plasmid to generate, e.g., pBS-H1-2095β (Scherr et al., 2003a). The correct sequence and insertion were confirmed by DNA sequencing for each plasmid. The controls pH1-GL4, pH1-121GFP, and pH1-243GFP were described earlier (Scherr et al., 2003a).

Construction of lentiviral vectors

pHR′-SIN-SEW (Demaison et al., 2002) was used to generate lentiviral transgenic plasmids containing H1-siRNA expression cassettes located in the U3 region of the Δ3′-LTR using an SnaBI-restriction site introduced by site-directed mutagenesis (Fig. 1B) (Scherr et al., 2003a). The location of the H1 expression cassette in the

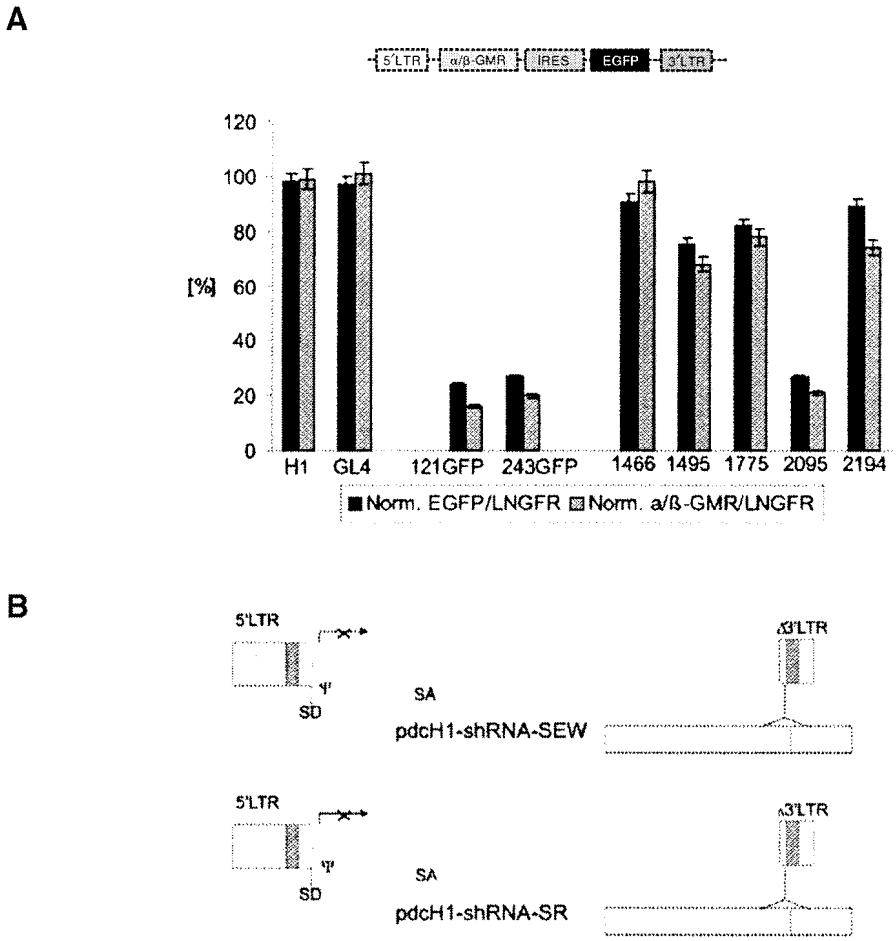

FIG. 1. siRNAs expressed from lentiviral vectors. (**A**) Flow cytometry analysis of α/β-GMR surface expression (stippled) and EGFP fluorescence (black) in BHK-21 cells cotransfected with a bicistronic expression plasmid encoding α/β-GMR and EGFP and individual shRNAs. LNGFR expression was used for normalization. H1, GL4, control shRNAs; 121GFP, 243GFP, anti-GFP shRNAs; 1466, 1495, 1775, 2095, 2194, anti-β-GMR shRNAs. (**B**) Schematic representation of the lentiviral transgene plasmids encoding shRNAs. The shRNA is transcribed from a human H1-RNA promoter inserted into the U3 region of the $\Delta 3'$-LTR of the lentiviral vector to obtain a double-copy vector. The vector encodes either EGFP or RFP as a marker gene driven by the spleen focus forming virus promoter (SSFV-LTR) and harbors a cPPT/CTS sequence. The WPRE element for enhanced transgene expression was only inserted into the pdcH1-shRNA-SEW transgene plasmid. 5′-LTR HIV-1 5′-LTR; $\Delta 3'$-LTR, HIV-1 self-inactivating (SIN) 3′-LTR, GA deleted *gag* sequence; RRE, rev-responsive element; SD, splice donor site; SA splice acceptor site; Ψ, packaging signal.

$\Delta 3'$-LTR results in duplication during reverse transcription, as described earlier (Scherr et al., 2003a). To generate double-copy (dc) H1-siRNA transgenic plasmids, the pH1-2095β and pH1-GL4 plasmids were digested with SmaI and HincII, and the resulting DNA fragments (360 nt) were blunt-end ligated into the SnaBI site of pHR′-SIN-SEW-SnaBI to generate pdcH1-2095β-SEW and pdcH1-GL4-SEW, as well as in the pHR′-SIN-SR-SnaBI to generate pdcH1-2095β-SR and pdcH1-GL4-SR, respectively (Scherr et al., 2003a). The SEW and SR lentiviral constructs encode enhanced green fluorescence protein (EGFP) and RFP as reporter genes, respectively.

Cell culture

The adherent cell lines BHK-21, 293T, and SC-1 were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), and 2 mM L-glutamine (GIBCO, Grand Island, NY). The cloning and functional characterization of α/β-GMR have been described earlier in detail (Eder et al., 1994; Kafert et al., 1999b). BaF3/α/β-GMR clones and BaF3/α/β-GMR-IRES-EGFP clones were generated as described, and individual clones were isolated by limiting dilution. The cells were grown in RPMI 1640 supplemented with 10% FBS and MuIL-3 (supplied as 10% conditioned medium from Wehi3B cells) or HuGM-CSF (20 ng/ml or as indicated).

Proliferation assay

GM-CSF-dependent proliferation was analyzed by trypan blue exclusion assay. Briefly, 5×10^4 BaF3/α/β-GMR cells/ml were grown in 96-well plates in a total volume of 200 μl with increasing amounts of HuGM-

CSF (0, 0.1, 0.5, 1, and 5 ng/ml). Cell numbers were analyzed after 72 hours by trypan blue exclusion.

Transfection

Transfections of BHK-21 cells were performed using the cationic lipid Lipofectamine 2000 (Life Technologies, Gaithersburg, MD). Cells (1×10^5) were seeded in a 24-well plate and transfected after 8 hours. The cotransfection was performed with three different plasmids: 1 μg MSCV-α/βGMR-IRES-GFP, 0.1 μg LNSN plasmid, encoding the low-affinity nerve growth factor receptor, and 1 μg shRNA expression vector, formulated into liposomes and added to cells as described by the manufacturer. After 48 hours, EGFP and low-affinity nerve growth factor receptor (LNGFR) expression was assessed by FACS and fluorescence microscopy (Axiovert 300, Nikon, Düsseldorf, Germany). To monitor LNGFR expression, 1×10^5 cells were incubated with phycoerythrin (PE) antihuman LNGFR antibody (mouse IgG) (PharMingen, San Diego, CA) for 30 minutes at 4°C, diluted with 1× phosphate-buffered saline (PBS), and collected by centrifugation for 10 minutes at 2000 rpm. To analyze α/β-GMR expression, 1×10^5 cells were incubated with a monoclonal antibody (mAb) directed against the extracellular domain of α-GMR (GM-CSFα S-20, dilution 1:100) (Santa Cruz Biotechnology, Santa Cruz, CA) for 60 minutes at 4°C, followed by incubation with an allophycocyamin (APC)-labeled goat anti-mouse antibody (Dianova, Hamburg, Germany).

Preparation of recombinant lentiviral supernatants and lentiviral transduction

VSV.G-pseudotyped lentiviral particles were generated by calcium phosphate cotransfection of 293T cells, and viral supernatants were concentrated as previously described (Scherr et al., 2002). dcH1-shRNA-SEW and dcH1-shRNA-SR lentiviral preparations were titered in triplicate by serial dilutions of the concentrated vector stocks on 1×10^5 SC-1 cells in 24-well plates. The number of EGFP-positive or RFP-positive cells was analyzed 72 hours posttransduction by FACS analysis (FACS-Calibur, Becton Dickinson, Mountain View, CA), typically yielding titers of about $1–5 \times 10^8$ IU/ml.

Lentiviral supernatants were used to transduce BaF3/α/β-GMR cells. Lentiviral transduction, including spin occulation, was performed as described earlier (Scherr et al., 2002). Two days after transduction, cells were expanded as bulk populations and analyzed by RT-PCR or FACS.

Real-time RT-PCR

Cytoplasmatic RNA was isolated using the RNeasy minispin columns (Qiagen, Hilden, Germany) from 1×10^6 BaF3/α/β-GMR cells and digested with 2 U DNase I (Roche, Mannheim, Germany) for 1 hour at 37°C, followed by phenol chloroform extraction and ethanol precipitation. RNA was reverse transcribed into cDNA in a total volume of 20 μl using MMLV-reverse transcriptase (Invitrogen, San Diego, CA) and random hexamer primers under standard conditions. Real-time Taqman RT-PCR of α/β-GMR and murine GAPDH was performed as described previously (Scherr et al., 2003b; Kafert et al., 1999a).

Northern blot analysis

Total RNA from transduced BaF3/α/β-GMR-IRES-GFP cells was isolated using Trizol according to the manufacturer's instruction (Invitrogen). RNA (20 μg) was subjected to 16% denaturing polyacrylamide gel electrophoresis and transferred onto Hybond-N+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ) by electroblotting. The hybridization and washing steps were carried out at 37°C. Membranes were probed with either ^{32}P-labeled 19-nt GFP or α/β-GMR oligonucleotide corresponding to the sense strand of the respective 121 and α/β-GMR siRNAs and were subsequently visualized by autoradiography. Endogenous U6 snRNA served both as an internal size standard and as a loading control (U6snRNA probe: 5′-TATGGAACGCTTCACGAATTTGC-3′).

Isolation and lentiviral transduction of peripheral blood-derived CD34$^+$ cells

G-CSF-primed CD34$^+$ cells were harvested by leukapheresis from four healthy volunteers, purified to ≥98% CD34$^+$ content by magnetic cell sorting (Clini MACS, Miltenyi Biotech, Bergisch Gladbach, Germany), and cryopreserved in liquid nitrogen. Lentiviral transduction of CD34$^+$ cells was performed twice as described previously (Scherr et al., 2002). Methylcellulose colony assays with SCF (20 ng/ml) + G-CSF (10 ng/ml) + TPO (10 U/ml) or GM-CSF (20 ng/ml) + IL-3 (10 ng/ml) were performed as described (Schiedlmeier et al., 2000). The cytokines were all from R&D Systems (Abingdon, U.K.).

Transplantation of CD34$^+$ cells into NOD/SCID mice

NOD/SCID mice were kept and transplanted with transduced and mock-transduced human CD34$^+$ cells (2×10^6 cells in 300 μl IMDM per mouse) and irradiated fibroblasts producing human IL-3 (Maingen, Frankfurt, Germany). Mice were killed 6 weeks after transplantation, and engraftment and multilineage RFP expression were analyzed by FACS (Schiedlmeier et al., 2000).

RESULTS

Selection of anti-β-GMR shRNAs

Several anti-β-GMR shRNAs were synthesized targeting 19 nt of the β-GMR sequence starting at β-GMR po-

sition 1466, 1495, 1775, 2095, and 2194, respectively, and were subsequently cloned into H1-shRNA expression cassettes. To quantify shRNA-triggered gene silencing, BHK-21 cells were cotransfected with (1) a bicistronic expression plasmid encoding a chimeric α/β-GMR identical to β-GMR 3′ of nucleotide 1424 (Eder et al., 1994) and EGFP (Fig. 1A), (2) an expression plasmid with the LNGFR cDNA used for normalization, and (3) individual shRNAs, including two anti-EGFP shRNAs as reported earlier (Scherr et al., 2003a). The chimeric α/β-GMR can be activated by human GM-CSF and mediates proliferation and survival in transfected cells, thus allowing study of GMR function on expression of a single transgene, as opposed to the wild-type α-GMR + β-GMR (Eder et al., 1994; Kafert et al., 1999b). As shown in Figure 1A, expression of both EGFP fluorescence and α/β-GMR was measured by FACS analysis 24 hours after cotransfection with each shRNA construct and normalized for LNGFR expression. The effects of individual shRNAs on both EGFP fluorescence and α/β-GMR surface expression were very similar in all cases. However, only one of five anti-β-GMR shRNAs (2095) inhibited target gene expression to an extent similar to both anti-EGFP shRNAs (up to 80%). In contrast, the remaining four β-GMR shRNAs induced only low or marginal inhibition of EGFP fluorescence and α/β-GMR surface expression.

The H1-2095 shRNA cassette was subsequently in-

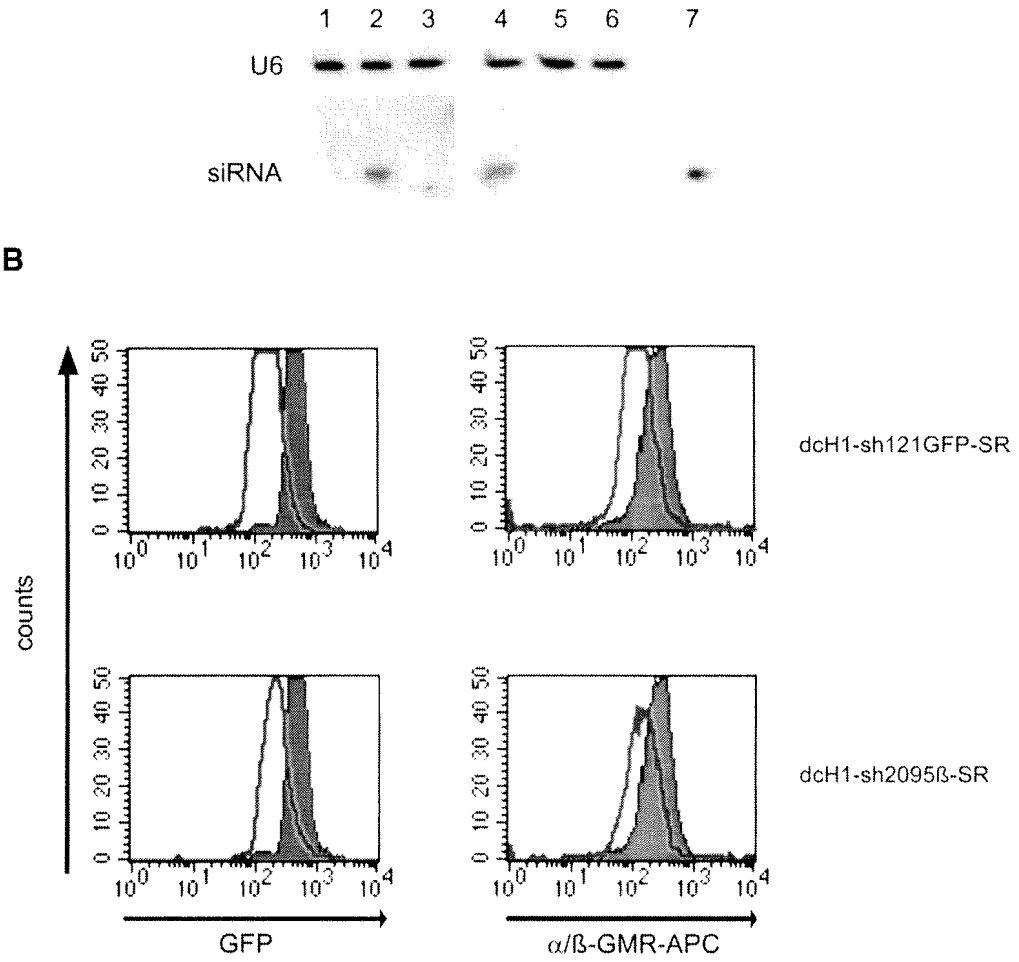

FIG. 2. Lentivirus-mediated gene silencing in BaF3-α/β-GMR-IRES-GFP cells. (**A**) Northern blot analysis depicts cellular siRNA expression after transduction of BaF3-α/β-GMR-IRES-GFP cells with dcH1-2095β-SR (lanes 2 and 5), dcH1-121GFP-SR (lanes 1 and 4), and dcH1-GL4-SR (lanes 3 and 6). Lane 7, a 21-nt long chemically synthesized 121 GFP siRNA duplex. The blot on the left (lanes 1–3) was probed with ^{32}P-labeled 19-nt β-GMR oligonucleotide corresponding to the sense strand of the 2095β siRNA, and the blots in the middle and on the right (lanes 4–7) were hybridized with ^{32}P-labeled 19-nt GFP oligonucleotide corresponding to the 121GFP siRNA. A shorter exposure to visualize U6 snRNA as loading control is shown in the upper panel. (**B**) Surface expression of α/β-GMR (right) and EGFP fluorescence (left) was measured at day 6 after a single transduction with either dcH1-121GFP-SR (**top**) or with dcH1-2095β-SR (**bottom**). The filled curves show untransduced BaF3-α/β-GMR-IRES-GFP cells as control.

serted into two lentiviral transgene plasmids to generate pdcH1-2095-SR and pdcH1-2095-SEW, respectively (Fig. 1B). Both constructs are optimized for lentiviral transduction by the cPPT/CTS sequence and harbor the H1-shRNA cassette located in the Δ3′-LTR, leading to their duplication during reverse transcription (dc constructs). pdcH1-2095-SR and pdcH1-2095-SEW differ in the reporter gene encoded (RFP and EGFP), and pdcH1-2095-SEW but not pdcH1-2095-SR contains a WPRE (W) element to enhance EGFP expression. Lentiviral supernatants were generated, and expression of siRNAs after lentiviral gene transfer was demonstrated by Northern blotting of RNA isolated from BaF3 cells expressing α/β-GMR and EGFP from the bicistronic transcript

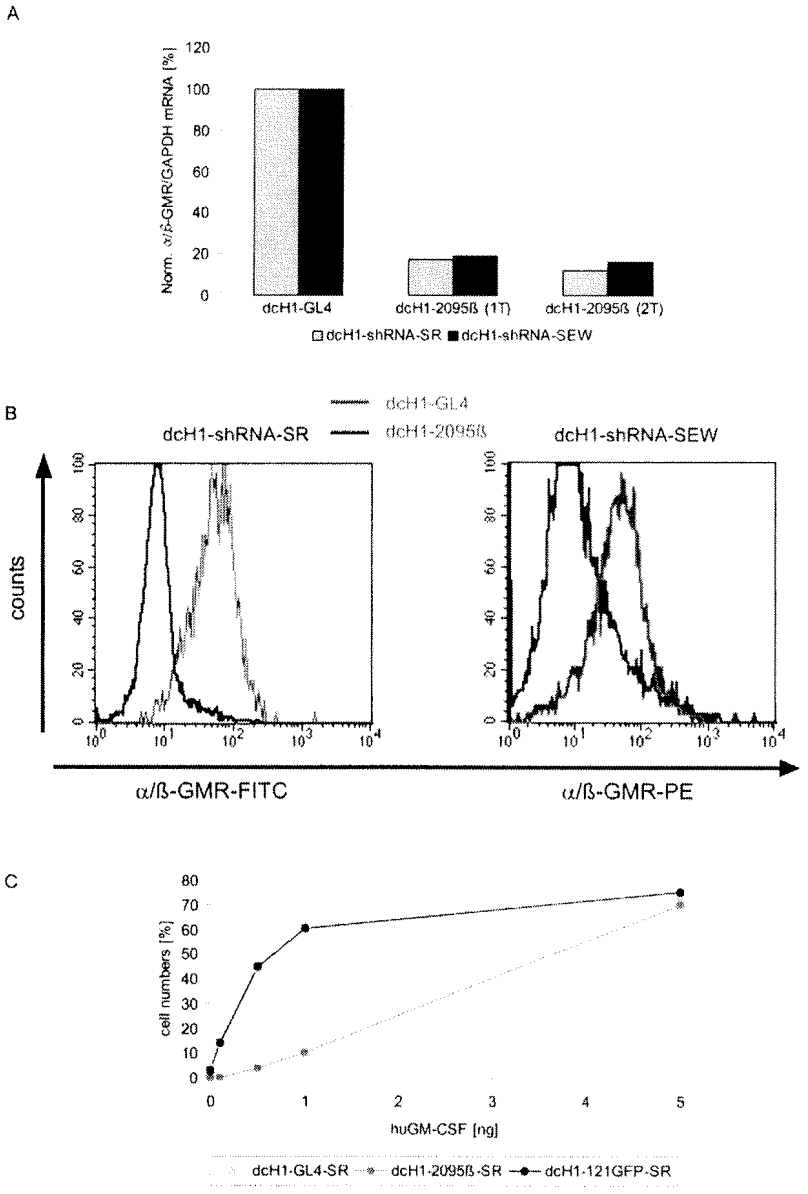

FIG. 3. Lentivirus-mediated gene silencing in BaF3-α/β-GMR cells (**A**) α/β-GMR mRNA levels were measured by real-time RT-PCR 6 days after repeated lentiviral transduction (IT, single transduction; 2T, double transduction) and were normalized in comparison to GAPDH expression. α/β-GMR expression after transduction with control shRNA dcH1-GL4 was set to 100%. dcH1-GL4-SEW and dcH1-GL4-SR encode control shRNA, and dcH1-2095β-SEW and dcH1-2095β-SR encode anti-β-GMR shRNAs. (**B**) Surface expression of α/β-GMR was measured at day 6 after the second transduction with either dcH1-shRNA-SR (**left**) or dcH1-shRNA-SEW (**right**) viruses. BaF3-α/β-GMR cells transduced with dcH1-GL4-SR (**left, panel, right curve**) or dcH1-GL4-SEW (**right panel, right curve**) served as controls. (**C**) Inhibition of GM-CSF-mediated cell proliferation in a dose-dependent manner (ranging from 0 to 5 ng/ml HuGM-CSF) by transduction with dcH1-2095-SR but not with control dcH1-GL4-SR or dcH1-121GFP-SR. Cell numbers of cultures stimulated with MuIL-3 were set 100%.

schematically shown in Figure 1A. As shown in Figure 2A, anti-β-GMR (lane 2) and anti-121GFP (lane 4) siRNAs can be generated from proviral dcH1-2095-SR and dcH1-121GFP-SR, respectively. In addition, equivalent inhibition of α/β-GMR surface expression and EGFP fluorescence mediated by either anti β-GMR or anti-121GFP siRNAs was observed in transduced BaF3α/β-GMR-IRES-EGFP cells (Fig. 2B).

Function of anti-β-GMR sh/siRNAs in a reporter cell line

To evaluate the efficiency of siRNA 2095β, BaF3 cells expressing α/β-GMR (BaF3/α/β-GMR) were transduced with either dcH1-2095-SR or dcH1-2095-SEW lentiviral supernatants. α/β-GMR mRNA levels were measured using real-time RT-PCR after one or two rounds of lentiviral transduction. As shown in Figure 3A, both lentiviral preparations inhibited target mRNA expression by about 80% compared with control shRNA. In addition, this decrease in target mRNA levels was not significantly enhanced by a second lentiviral transduction. FACS analysis of α/β-GMR protein expression on the cell surface revealed a marked and slightly higher reduction on transduction with dcH1-2095-SR compared with dcH1-2095-SEW (Fig. 3B). Interestingly, >95% of BaF3/α/β-GMR cells exhibited green and red fluorescence, respectively, indicating a 95% or higher transduction rate (data not shown). Based on these results, all further experiments were performed with dcH1-2095-SR lentiviral preparations.

To analyze RNAi-mediated functional defects in α/β-GMR signaling, BaF3/α/β-GMR cells were cultured in medium containing different concentrations of HuGM-CSF. BaF3 cells are murine pro-B cells that require MuIL-3 for survival and proliferation. However, expression of α/β-GMR enables culture in either MuIL-3 or HuGM-CSF (Eder et al., 1994). As shown in Figure 3C, transduction with dcH1-2095-SR, but not with control dcH1-GL4-SR or dcH1-121GFP-SR, inhibited GM-CSF-mediated cell proliferation in a dose-dependent manner. As BaF3/α/β-GMR cells express α/β-GMR from a monocistronic transcript that does not contain EGFP, anti-GFP siRNA had no effect on α/β-GMR gene expression in these cells.

Function of anti-β-GMR sh/siRNAs in primary CD34$^+$ SRC and progenitor cells

To analyze RNAi in HSC and their progeny, lentivirally transduced CD34$^+$ cells were transplanted into NOD/SCID mice. The multilineage engraftment of lentivirally transduced human CD34$^+$ cells harvested 6

TABLE 1. LYMPHOHEMATOPOIETIC RECONSTITUTION OF NOD/SCID MICE[a]

	Mouse 1 dcH1-GL4-SR	*Mouse 2 dcH1-GL4-SR*	*Mouse 3 dcH1-2095β-SR*	*Mouse 4 dcH1-2095β-SR*	*Mouse 5 dcH1-2095β-SR*
HuCD45$^+$	87	58	75	94	60
RFP$^+$/HuCD45$^+$	8	8	10	8	7
CD34$^+$/HuCD45$^+$	3	3	3	4	3
RFP$^+$/CD34$^+$	NE[b]	NE	NE	NE	NE
CD33$^+$/HuCD45$^+$	65	70	70	69	66
RFP$^+$/CD33$^+$	7	9	13	7	11
CD38$^+$/HuCD45$^+$	61	55	58	63	62
RFP$^+$/CD38$^+$	11	13	14	9	10
CD19$^+$/HuCD45$^+$	32	28	28	27	33
RFP$^+$/CD19$^+$	6	6	5	3	3
CD14$^+$/HuCD45$^+$	6	9	8	5	9
RFP$^+$/CD14$^+$	NE	NE	NE	NE	NE

[a]The ratio of human cells (HuCD45$^+$), lymphohematopoietic subpopulations (CD34$^+$, CD33$^+$, CD38$^+$, CD19$^+$, and CD14), and the level of RFP expression are given in percent. RFP$^+$/CD45$^+$ represents the percentage of RFP$^+$ cells within the human CD45$^+$ cell fraction.

[b]NE, not evaluable; subpopulations with <10% engraftment were considered not suitable for accurate analysis of lentiviral transduction.

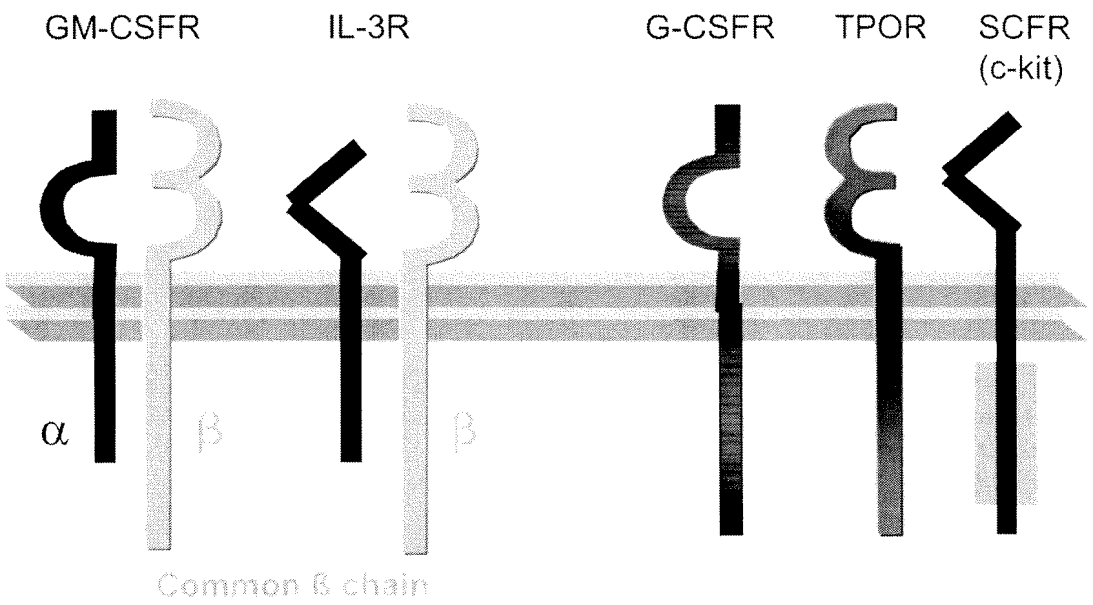

FIG. 4. Hematopoietic cytokine receptors. Illustrated here are the human GM-CSF and IL-3 receptors with the common β-chain at left and the receptors for G-CSF, TPO, and SCF at right. The shaded box in SCFR (c-*kit*) indicates its cytoplasmic tyrosine kinase domain.

weeks after transplantation is shown in Table 1. Analysis of human cells in murine bone marrow revealed no difference between mice engrafted with dcH1-2095-SR or dcH1-GL4-SR transduced $CD34^+$ cells with 58%–94% of all nucleated bone marrow cells of human origin. In addition, the ratio of transduced, RFP+ cells in the myeloid and B lymphoid compartment was as high as 10% in human $CD45^+$ cells and was similar in control and anti-β-GMR siRNA mice.

To study functional gene silencing, clonogenic assays of hematopoietic progenitor cells were performed before and after transplantation of $CD34^+$ cells into NOD/SCID mice. This *in vitro* culture allows functional analysis of specific cytokine receptors depending on the cytokines used for stimulation. As schematically shown in Figure 4, stimulation with GM-CSF and IL-3 requires functional β-GMR, whereas signal transduction initiated by G-CSF, SCF, and TPO is independent of β-GMR. Accordingly, normal $CD34^+$ cells transduced with control dcH1-GL4-SR lentivirus generate RFP^+ and RFP^- colonies at a nearly identical ratio under stimulation with either GM-CSF/IL-3 or G-CSF/SCF/TPO, with transduction rates between 5% and 18% in the G-CSF/SCF/TPO condition, which is not affected by RNAi against β-GMR (Table 2A). In contrast, the ratio of RFP^+ colonies derived from $CD34^+$ cells transduced with dcH1-2095-SR decreased by an average of about 60% when cultured in the presence of GM-CSF and IL-3, compared with G-CSF/SCF/TPO. The transduction rates after infection with dcH1-2095-SR were higher than those observed with dcH1-GL4-SR and ranged between 11% and 52% for individual donors (determined under stimulation with G-CSF/SCF/TPO).

Identical clonogenic cultures were performed with cells harvested from bone marrow of individual NOD/SCID mice engrafted with either dcH1-2095-SR or dcH1-GL4-SR-transduced $CD34^+$ cells. Again, the ratio of RFP^+ colonies was similar for both cytokine conditions in the dcH1-GL4-SR control mice (Table 2B). In contrast, the ratio of RFP-transduced colonies decreased by an average of 62% under stimulation with GM-CSF/IL-3 compared with G-CSF/SCF/TPO in the three mice engrafted with dcH1-2095-SR-transduced $CD34^+$ cells.

DISCUSSION

We analyzed GM-CSF receptor function to study RNAi in primary human $CD34^+$ hematopoietic stem and progenitor cells. We selected anti-β-GMR shRNAs based on their capacity to reduce target mRNA levels in a transient cotransfection assay. This approach uses bicistronic transcripts and allows rapid and quantitative detection of RNAi by FACS analysis of EGFP fluorescence. In our experiments, RNAi induced an equivalent reduction of both mRNAs of the bicistronic transcript independent of the 5′ or 3′ location of the respective target gene for RNAi. Only one of five anti-β-GMR shRNA, but both pretested anti-GFP shRNAs (Scherr et al., 2003a) induced effective gene silencing in this assay.

Lentiviral gene transfer was used to stably introduce H1-shRNA expression cassettes into the target cell genome. Constitutive transcription of shRNAs can induce long-term gene silencing, as demonstrated in earlier studies (Scherr et al., 2003a). The anti-β-GMR shRNA was

TABLE 2A. COLONY FORMATION OF CD34$^+$ HEMATOPOIETIC PROGENITOR CELLS[a]

	CD34$^+$ sample	*Number RFP$^+$ colonies/total colony number(%)*	
		GM-CSF/IL-3	*G-CSF/SCF/TPO*
dcH1-GL4-SR	A	11	10
dcH1-2095β-SR		3	11
dcH1-GL4-SR	B.1	16	18
dcH1-2095β-SR		32	52
dcH1-GL4-SR	B.2	15	17
dcH1-2095β-SR		6	32
dcH1-GL4-SR	C	4	5
dcH1-2095β-SR		7	24
dcH1-GL4-SR	D	19	18
dcH1-2095β-SR		19	32

[a]CD34$^+$ samples enumerated in alphabetical order. Sample B was transduced with different lentiviral vector preparations.

TABLE 2B. COLONY FORMATION OF SRC-DERIVED HEMATOPOIETIC PROGENITOR CELLS

Transplanted mice	*Sample*	*Number RFP$^+$ colonies/Total colony number*	
		GM-CSF/IL-3	*G-CSF/SCF/TPO*
Mouse 1	dcH1-GL4-SR	10	8
Mouse 2	dcH1-GL4-SR	7	9
Mouse 3	dcH1-2095β-SR	9	15
Mouse 4	dcH1-2095β-SR	4	14
Mouse 5	dcH1-2095β-SR	3	11

tested in a murine cell line that expresses a chimeric single chain α/β-GMR, which encodes the entire intracellular domain of β-GMR. A significant RNAi-mediated reduction in target mRNA and surface protein expression was found in BaF3/α/β-GMR cells. In addition, RNAi inhibited α/β-GMR function depending on the GM-CSF dose. Interestingly, these results correspond very well to the inhibition of α/β-GMR function observed on coexpression of a dominant negative splice variant of β-GMR in BaF3/α/β-GMR cells (Wagner et al., 2001), indicating that higher doses of ligand can compensate for lower numbers of functional receptor molecules in this specific model.

We next examined the engraftment and differentiation capacity of human primary CD34$^+$ cells after lentiviral transduction with anti-β-GMR shRNA expression cassettes in the NOD/SCID mouse model. This model is considered a standard tool to analyse SRC as a surrogate for human HSC (Dick et al., 1997). Our data indicate that lentivirus-mediated RNAi targeting the endogenous β-GMR gene did not significantly affect engraftment and lineage composition of hematopoietic cells compared with controls. This is not due to loss of RNAi or transient gene silencing, as the phenotype of β-GMR inhibition remains almost identically conserved in colony-forming cells before and after transplantation, demonstrating long-term functional gene silencing (Tables 2A and 2B). These data may indicate that β-GMR function is not required for engraftment of SRC, as suggested by the fact that many groups omit *in vivo* treatment with HuIL-3 without negative effects on SRC engraftment. Alternatively, transduced cells that express sufficient β-GMR in spite of RNAi may be selected under the treatment conditions used in this study. A higher number of NOD/SCID mice have to be analyzed using different treatment modalities to definitively characterize potential functions of β-GMR in the NOD/SCID xenotransplantation model. Furthermore, we demonstrate that RFP can be used as a reporter gene for lentiviral transduction in the NOD/SCID model. Future studies are necessary to compare the effects of different reporter genes, such as RFP or EGFP, on SRC or hematopoietic progenitor cells.

In summary, our data demonstrate for the first time that

lentiviral transfer of suitable shRNA expression cassettes can induce long-term RNAi in primary SRC without detectable effects on engraftment capability and lineage composition of SRC-derived hematopoietic cells. This approach will allow functional genomics in primary human HSC and their myeloid progeny as demonstrated here for β-GMR. Finally, stable RNAi may eventually be applied as a therapeutic strategy in hematologic diseases characterized by aberrant gene expression either due to mutations as in cancer or derived from foreign infectious organisms.

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REFERENCES

ABBAS-TERKI, T., BLANCO-BOSE, W., DEGLON, N., PRALONG, W., and AEBISCHER, P. (2002). Lentiviral-mediated RNA interference. Hum. Gene Ther. **13,** 2197–2201.

ASHRAFI, K., CHANG, F.Y., WATTS, J.L., FRASER, A.G., KAMATH, R.S., AHRINGER, J., et al. (2003). Genome-wide RNAi analysis of *Caenorhabditis elegans* fat regulatory genes. Nature **421,** 268–272.

BANERJEA, A., LI, M.J., BAUER, G., REMLING, L., LEE, N.S., ROSSI, J., and AKKINA, R. (2003). Inhibition of HIV-1 by lentiviral vector-transduced siRNAs in T lymphocytes differentiated in SCID-hu mice and CD34$^{(+)}$ progenitor cell-derived macrophages. Mol. Ther. **8,** 62–71.

BARTON, G.M., and MEDZHITOV, R. (2002). Retroviral delivery of small interfering RNA into primary cells. Proc. Natl. Acad. Sci. USA **99,** 14943–14945.

BRUMMELKAMP, T.R., BERNARDS, R., and AGAMI, R. (2002). Stable suppression of tumorigenicity by virus-mediated RNA interference. Cancer Cell **2,** 243–247.

CAPODICI, J., KARIKO, K., and WEISSMAN, D. (2002). Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference. J. Immunol. **169,** 5196–5201.

CATALANOTTO, C., AZZALIN, G., MACINO, G., and COGONI, C. (2000). Gene silencing in worms and fungi. Nature **404,** 245.

COBURN, G.A., and CULLEN, B.R. (2002). Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. J. Virol. **76,** 9225–9231.

DEMAISON, C., PARSLEY, K., BROUNS, G., SCHERR, M., BATTMER, K., KINNON, C., GREZ, M., and THRASHER, A.J. (2002). High-level transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency virus type 1-based lentiviral vector containing an internal spleen focus forming virus promoter. Hum. Gene Ther. **13,** 803–813.

DEVROE, E., and SILVER, P.A. (2002). Retrovirus-delivered siRNA. BMC Biotechnol. **2,** 7–15.

DICK, J.E., BHATIA, M., GAN, O., KAPP, U., and WANG, J.C. (1997). Assay of human stem cells by repopulation of NOD/SCID mice. Stem Cells **1,** 199–203.

EDER, M., ERNST, T.J., GANSER, A., JUBINSKY, P.T., INHORN, R., HOELZER, D., and GRIFFIN, J.D. (1994). A low affinity chimeric human alpha/beta-granulocyte-macrophage colony-stimulating factor receptor induces ligand-dependent proliferation in a murine cell line. J. Biol. Chem. **269,** 30173–30180.

ELBASHIR, S.M., HARBORTH, J., LENDECKEL, W., YALCIN, A., WEBER, K., and TUSCHL, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature **411,** 494–498.

FIRE, A., XU, S., MONTGOMERY, M.K., KOSTAS, S.A., DRIVER, S.E., and MELLO, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans.* Nature **391,** 806–811.

FRASER, A.G., KAMATH, R.S., ZIPPERLEN, P., MARTINEZ-CAMPOS, M., SOHRMANN, M., and AHRINGER, J. (2000). Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. Nature **408,** 325–330.

GONCZY, P., ECHEVERRI, C., OEGEMA, K., COULSON, A., JONES, S.J., COPLEY, R.R., et al. (2000). Functional genomic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III. Nature **408,** 331–336.

HAYASHIDA, K., KITAMURA, T., GORMAN, D.M., ARAI, K., YOKOTA, T., and MIYAJIMA, A. (1990). Molecular cloning of a second subunit of the receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF): Reconstitution of a high-affinity GM-CSF receptor. Proc. Natl. Acad. Sci. USA **87,** 9655–9659.

IHLE, J.N. (1995). Cytokine receptor signalling. Nature **377,** 591–594.

JACQUE, J.M., TRIQUES, K., and STEVENSON, M. (2002). Modulation of HIV-1 replication by RNA interference. Nature **418,** 435–438.

KAFERT, S., KRAUTER, J., GANSER, A., and EDER, M. (1999a). Differential quantitation of alternatively spliced messenger RNAs using isoform-specific real-time RT-PCR. Anal. Biochem. **269,** 210–213.

KAFERT, S., LUTHER, S., BOLL, I., WAGNER, K., GANSER, A., and EDER, M. (1999b). Functional analysis of a single chain chimeric alpha/beta-granulocyte-macrophage colony-stimulating factor receptor. Importance of a glutamate residue in the transmembrane region. J. Biol. Chem. **274,** 33064–33071.

KAMATH, R.S., FRASER, A.G., DONG, Y., POULIN, G., DURBIN, R., GOTTA, M., et al. (2003). Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. Nature **421,** 231–237.

KUNATH, T., GISH, G., LICKERT, H., JONES, N., PAWSON, T., and ROSSANT, J. (2003). Transgenic RNA interference in ES cell-derived embryos recapitulates a genetic null phenotype. Nat. Biotechnol. **21,** 559–561.

MARTINEZ, L.A., NAGUIBNEVA, I., LEHRMANN, H., VERVISCH, A., TCHENIO, T., LOZANO, G., and HAREL-BELLAN, A. (2002). Synthetic small inhibiting

RNAs: Efficient tools to inactivate oncogenic mutations and restore p53 pathways. Proc. Natl. Acad. Sci. USA **99,** 14849–14854.

McMANUS, M.T., HAINES, B.B., DILLON, C.P., WHITHURST, C.E., VAN PARIJS, L., CHEN, J., and SHARP, P.A. (2002). Small interfering RNA-mediated gene silencing in T lymphocytes. J. Immunol. **169,** 5754–5760.

METCALF, D. (1989). The molecular control of cell division, differentiation commitment and maturation in hematopoietic cells. Nature **339,** 27–30.

NGO, H., TSCHUDI, C., GULL, K., and ULLU, E. (1998). Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei.* Proc. Natl. Acad. Sci. USA **95,** 14687–14692.

PAL-BHADRA, M., BHADRA, U., and BIRCHLER, J.A. (2002). RNAi-related mechanisms affect both transcriptional and posttranscriptional transgene silencing in *Drosophila.* Mol. Cell **9,** 315–327.

QIN, X.F., AN, D.S., CHEN, I.S., and BALTIMORE, D. (2003). Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. Proc. Natl. Acad. Sci. USA **100,** 183–188.

ROIGNANT, J.Y., CARRE, C., MUGAT, B., SZYMCZAK, D., LEPESANT, J.A., and ANTONIEWSKI, C. (2003). Absence of transitive and systemic pathways allows cell-specific and isoform-specific RNAi in *Drosophila.* RNA **9,** 299–308.

RUBINSON, D.A., DILLON, C.P., KWIATKOWSKI, A.V., SIEVERS, C., YANG, L., KOPINJA, J., ZHANG, M., McMANUS, M.T., GERTLER, F.B., SCOTT, M.L., and VAN PARIJS, L. (2003). A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat. Genet. **33,** 401–406.

SCHERR, M., BATTMER, K., BLOMER, U., SCHIEDLMEIER, B., GANSER, A., GREZ, M., and EDER, M. (2002). Lentiviral gene transfer into peripheral blood-derived CD34$^+$ NOD/SCID-repopulating cells. Blood **99,** 709–712.

SCHERR, M., BATTMER, K., GANSER, A., and EDER, M. (2003a). Modulation of gene expression by lentiviral-mediated delivery of small interfering RNA. Cell Cycle **2,** 251–257.

SCHERR, M., BATTMER, K., WINKLER, T., HEIDENREICH, O., GANSER, A., and EDER, M. (2003b). Specific inhibition of *bcr-abl* gene expression by small interfering RNA. Blood **101,** 1566–1569.

SCHIEDLMEIER, B., KUHLCKE, K., ECKERT, H.G., BAUM, C., ZELLER, W.J., and FRUEHAUF, S. (2000). Quantitative assessment of retroviral transfer of the human multidrug resistance 1 gene to human mobilized peripheral blood progenitor cells engrafted in nonobese diabetic/severe combined immunodeficient mice. Blood **95,** 1237–1248.

STEWART, S.A., DYKXHOORN, D.M., PALLISER, D., MIZUNO, H., YU, E.Y., AN, D.S., SABATINI, D.M., CHEN, I.S., HAHN, W.C., SHARP, P.A., WEINBERG, R.A., and NOVINA, C.D. (2003). Lentivirus-delivered stable gene silencing by RNAi in primary cells. RNA **9,** 493–501.

WAGNER, K., KAFERT-KASTING, S., HEIL, G., GANSER, A., and EDER, M. (2001). Inhibition of granulocyte-macrophage colony-stimulating factor receptor function by a splice variant of the common beta-receptor subunit. Blood **98,** 2689–2696.

WIANNY, F., and ZERNICKA-GOETZ, M. (2000). Specific interference with gene function by double-stranded RNA in early mouse development. Nat. Cell. Biol. **2,** 70–75.

XIA, H., MAO, Q., PAULSON, H.L., and DAVIDSON, B.L. (2002). siRNA-mediated gene silencing *in vitro* and *in vivo.* Nat. Biotechnol. **20,** 1006–1010.

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RNA interference targeting Fas protects mice from fulminant hepatitis

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RNA interference (RNAi) is a powerful tool to silence gene expression post-transcriptionally[1]. However, its potential to treat or prevent disease remains unproven. Fas-mediated apoptosis is implicated in a broad spectrum of liver diseases, where inhibiting hepatocyte death is life-saving[2]. We investigated the *in vivo* silencing effect of small interfering RNA (siRNA) duplexes targeting the gene *Fas* (also known as *Tnfrsf6*), encoding the Fas receptor, to protect mice from liver failure and fibrosis in two models of autoimmune hepatitis. Intravenous injection of *Fas* siRNA specifically reduced *Fas* mRNA levels and expression of Fas protein in mouse hepatocytes, and the effects persisted without diminution for 10 days. Hepatocytes isolated from mice treated with *Fas* siRNA were resistant to apoptosis when exposed to Fas-specific antibody or co-cultured with concanavalin A (ConA)-stimulated hepatic mononuclear cells. Treatment with *Fas* siRNA 2 days before ConA challenge abrogated hepatocyte necrosis and inflammatory infiltration and markedly reduced serum concentrations of transaminases. Administering *Fas* siRNA beginning one week after initiating weekly ConA injections protected mice from liver fibrosis. In a more fulminant hepatitis induced by injecting agonistic Fas-specific antibody, 82% of mice treated with siRNA that effectively silenced *Fas* survived for 10 days of observation, whereas all control mice died within 3 days. Silencing Fas expression with RNAi holds therapeutic promise to prevent liver injury by protecting hepatocytes from cytotoxicity.

RNAi by synthetic siRNAs 21–23 nucleotides in length silences cellular and viral gene expression in mammalian cells *in vitro*[3,4]. siRNA duplexes pulse-injected into the tail vein of mice inhibit expression of a co-transfected firefly luciferase gene[5,6]. Viral-mediated delivery of siRNA *in vivo* reduces exogenous green fluorescent protein (GFP) and endogenous glucuronidase expression[7]. In these studies, RNA silencing was prominent in the liver, indicating that the liver is an ideal organ to test the therapeutic potential of siRNA. Hepatocytes are very susceptible to Fas-mediated apoptosis because they highly express Fas[8]. As a consequence, Fas-mediated apoptosis is important in hepatic injury from diverse insults, including viruses, autoimmunity and transplant rejection[2,9]. Fas-deficient *lpr* mice survive challenge with factors that induce fulminant hepatitis in normal mice [10,11] and show reduced fibrosis after chronic hepatic insults[12]. Therefore, we investigated whether intravenous siRNA injection targeting *Fas* could inhibit Fas expression on mouse hepatocytes *in vivo* and protect the liver from fulminant hepatitis and fibrosis.

First, we verified delivery of synthetic siRNA duplexes into mouse hepatocytes *in vivo* by hydrodynamic tail vein injection of Cy5-labeled *Fas*(sequence 1) siRNA (50 μg, 2.0–2.5 mg/kg)[13] (for details of *Fas* target sequences, see Methods). Twenty-four hours after the last of three injections, 88 ± 6% of hepatocytes had taken up the siRNA and were Cy5-positive as determined by flow cytometry (Fig. 1*a*). The efficient delivery confirms that siRNA duplexes can be taken up by most liver cells *in vivo*[5,6]. Transduction efficiency was higher than the 40% efficiency observed with a single injection of reporter plasmid DNA[13]. Measuring uptake of Cy5–*Fas* siRNA is only a surrogate for measuring *Fas* siRNA uptake *in vivo*, however, because siRNA labeled with Cy5 at the 3′ end does not induce silencing (data not shown). *Fas* mRNA and protein expression in hepatocytes was measured by RNase protection assay (RPA) and immunoblotting, respectively, at various times after injection. Treatment with *Fas*(sequence 1) siRNA reduced *Fas* mRNA expression eight to tenfold as compared to saline or *GFP* siRNA injection (ratios of *Fas* signal to *Gapdh* signal (internal control): 0.0024 ± 0.0004 versus 0.022 ± 0.002 saline or 0.022 ± 0.002 *GFP* (sequence 1) siRNA, $n = 3$ per group, $P < 0.001$ compared with either control), as measured 24 h after the last injection (Fig. 1*b*). Immunoblot analysis showed that *Fas*(sequence 1) siRNA reduced Fas protein in hepatocytes nearly to background (Fig. 1*c*). The effect was specific, as injection of control siRNA targeting *GFP* did not change Fas expression, and *Fas*(sequence 1) siRNA treatment did not affect the expression of other *Fas*-related genes, such as those encoding FasL, FADD, FAF, TRAIL and TNF receptor p55 and RIP (Fig. 1*b*). Specificity was also demonstrated by RPAs of hepatocytes from mice injected with siRNAs targeting other regions of *Fas*. Two additional siRNAs, sequences 5 and 6, silenced Fas expression by ~81–86%, as efficiently as sequence 1. In contrast, sequence 2, beginning only one nucleotide downstream of sequence 6, reduced Fas expression by only 38% (Fig. 3*e*). Two sequences (3 and 4) did not suppress Fas expression at all.

siRNA seemingly suppresses expression more efficiently than does the intraperitoneal injection of antisense oligodeoxynucleotide (ODN) described in a previous report[14], where mice were treated with a higher dosage (6 mg/kg) of anti-*Fas* ODN for 12 consecutive days. Approximately 14-fold less total nucleic acid was administered here (50 μg for 3 injections to mice weighing

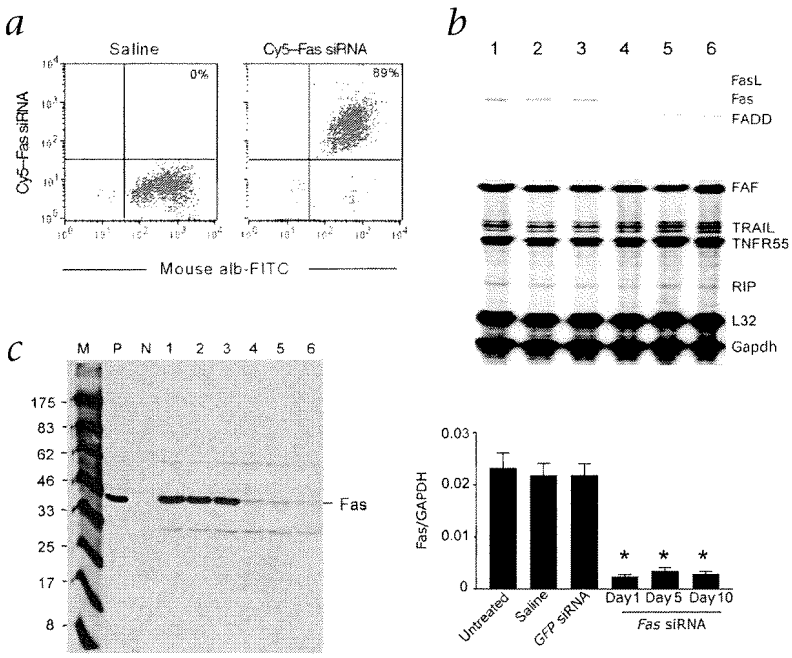

Fig. 1 Injection of siRNA duplex efficiently silences *Fas* gene expression in mouse hepatocytes. ***a***, Hepatocytes harvested 24 h after 3 injections of saline or Cy-5-labeled *Fas*(sequence 1) siRNA were stained with albumin-FITC (alb-FITC) and analyzed by flow cytometry. A high proportion of hepatocytes take up the duplex siRNA, as indicated. ***b***, RPA for *Fas* mRNA expression in hepatocytes from mice that were untreated (lane 1) or were injected 24 h earlier with saline (lane 2), *GFP*(sequence 1) siRNA (lane 3) or *Fas*(sequence 1) siRNA (lane 4). Silencing of *Fas* expression in mice treated with *Fas* siRNA is maintained 5 (lane 5) or 10 d (lane 6) later. Expression of other genes involved in the Fas pathway and housekeeping genes (*L32* and *Gapd*) were unaffected (names of the corresponding proteins are listed at right). Similar results were obtained in 3 independent experiments. The graph shows results of densitometric quantification of the *Fas*/*Gapdh* ratios in 3 mice per condition. *Fas* mRNA levels in hepatocytes are significantly lower (*, $P < 0.001$) at all times in mice treated with *Fas* siRNA mice than in control mice. ***c***, Fas immunoblot of lysates from hepatocytes obtained from untreated mice (lane 1), or 24 h after saline (lane 2), *GFP* siRNA (lane 3) or *Fas* siRNA (lane 4) injection, and 5 (lane 5) or 10 d (lane 6) after *Fas* siRNA injection. Mouse recombinant Fas and FasL proteins serve as positive (P) and negative (N) controls, respectively. Similar results were obtained in 3 independent experiments.

~24 g) to reduce Fas expression to a similar degree. In a recent study, siRNA was quantitatively more efficient than antisense ODN at suppressing co-transfected GFP expression both *in vitro* and *in vivo*[15]. However, a direct comparison using varying doses is needed to determine relative efficiency.

A major concern in applying siRNA therapeutically is the stability of silencing under physiological conditions. Double-stranded siRNAs resist biodegradation in fetal calf serum and in human plasma[15], and in one study siRNA-directed suppression of a co-transfected gene *in vivo* in mouse liver was maintained for several days[6]. In our study, levels of both *Fas* mRNA and Fas protein were stably reduced for 10 days after the last injection (*Fas*/*Gapdh* mRNA ratios: day 1, 0.0024 ± 0.0004; day 5, 0.0035 ± 0.0006; day 10, 0.0029 ± 0.0004, $P > 0.05$) (Fig. 1*b* and *c*). *Fas* mRNA levels and Fas protein expression were still only 40% of those in control mice on day 14, but returned to normal 20 days after the last injection (data not shown). The duration of silencing in hepatocytes, which contrasts with more transient silencing in transformed cell lines[3], indicates that sustained therapeutic silencing in hepatocytes may not require siRNA expression from plasmids or viral vectors. The difference between liver cells and cell lines probably occurs because hepatocytes are mostly non-dividing, so there is no siRNA dilution with cell division. However, although unlikely[16], the possibility that sustained suppression in hepatocytes might be due to siRNA amplification, which occurs in lower species[17,18], has not been ruled out. Because silencing after duplex siRNA injection is prolonged but not permanent, long-term toxicity, such as lymphoproliferative or autoimmune disease, seen in humans with mutations of the homolog *FAS* (*TNFRSF6*) and in the *lpr* mouse[19] is probably of little concern.

Two known mechanisms underlie Fas-mediated fulminant hepatitis. Ligation of the Fas receptor on hepatocytes induces massive apoptosis, accompanied by an infiltration of inflammatory cells and secondary necrosis[20]. Fas engagement also provokes hepatic inflammation by inducing expression of hepatic chemokines that recruit and activate immune cells, leading to hepatocyte death in a pro-inflammatory milieu[21]. To determine whether the efficient suppression of Fas

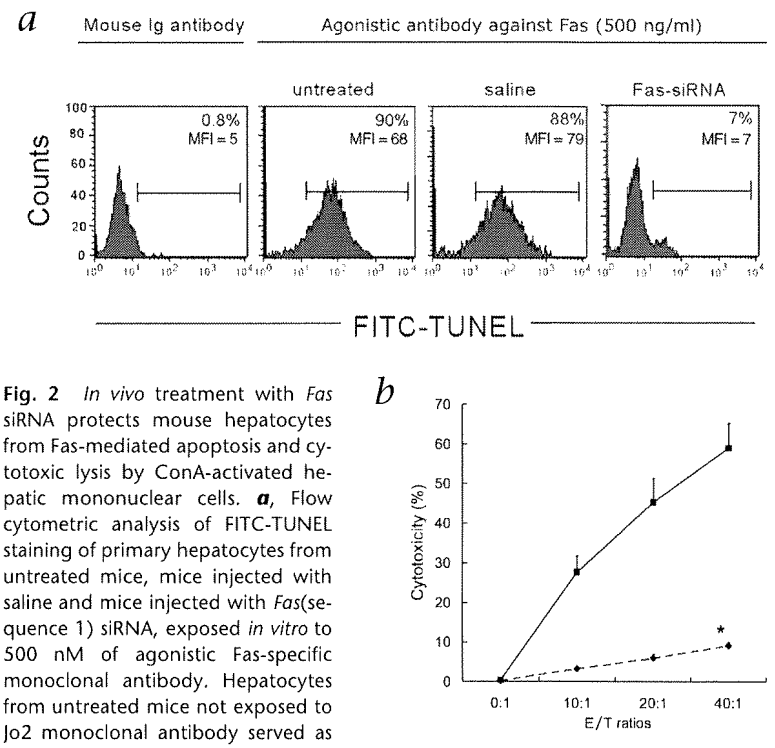

Fig. 2 *In vivo* treatment with *Fas* siRNA protects mouse hepatocytes from Fas-mediated apoptosis and cytotoxic lysis by ConA-activated hepatic mononuclear cells. ***a***, Flow cytometric analysis of FITC-TUNEL staining of primary hepatocytes from untreated mice, mice injected with saline and mice injected with *Fas*(sequence 1) siRNA, exposed *in vitro* to 500 nM of agonistic Fas-specific monoclonal antibody. Hepatocytes from untreated mice not exposed to Jo2 monoclonal antibody served as negative control. Percentage of FITC-TUNEL-positive cells and mean fluorescence intensity (MFI) are indicated. ***b***, Hepatic mononuclear cells from ConA-injected mice lyse hepatocytes from mice treated with saline (■) but not mice injected with *Fas*(sequence 1) siRNA (◆). E/T ratio, effector/target ratio. Similar results were obtained in 3 independent experiments. *, $P < 0.001$ at each ratio.

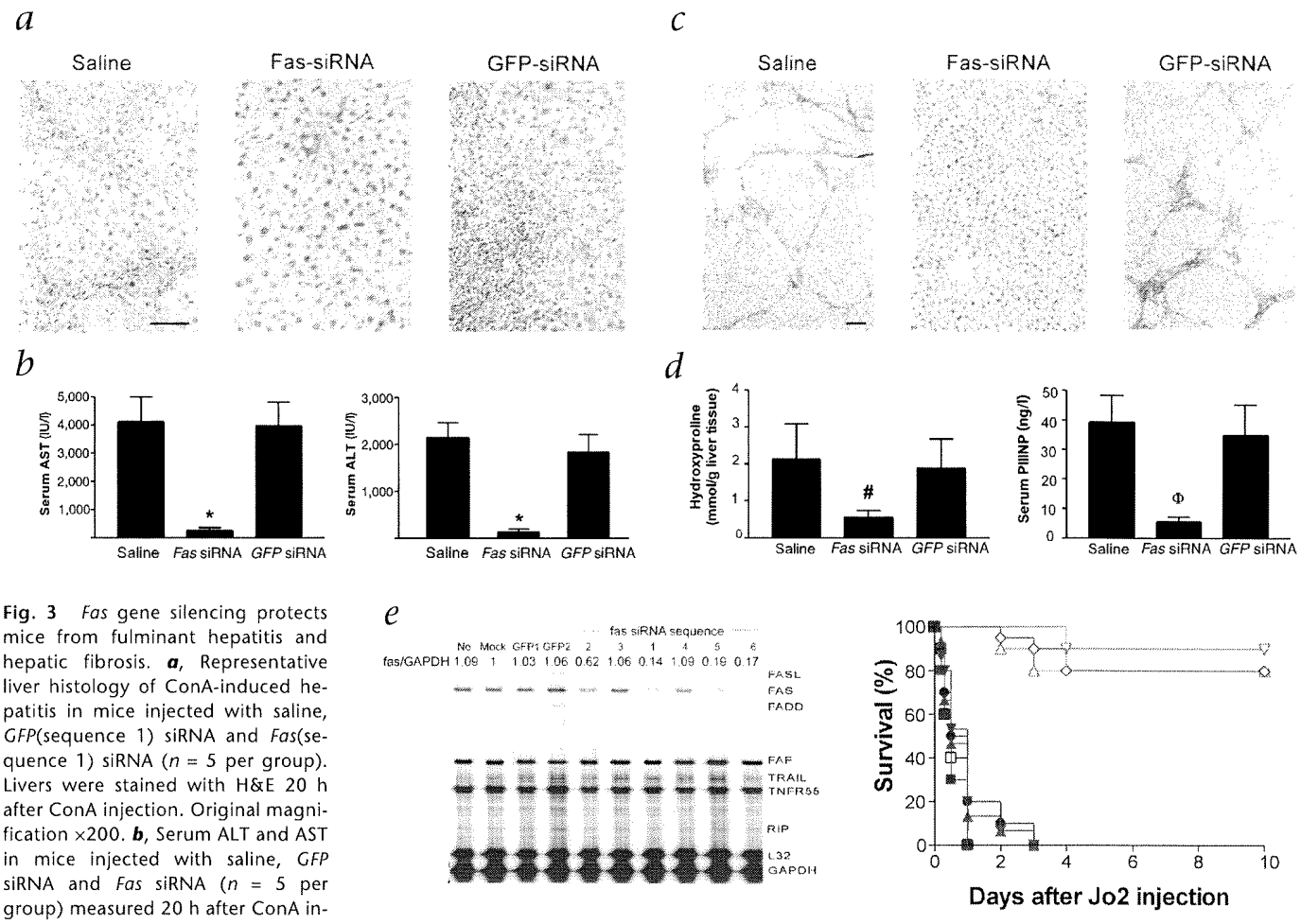

Fig. 3 *Fas* gene silencing protects mice from fulminant hepatitis and hepatic fibrosis. ***a***, Representative liver histology of ConA-induced hepatitis in mice injected with saline, *GFP*(sequence 1) siRNA and *Fas*(sequence 1) siRNA (n = 5 per group). Livers were stained with H&E 20 h after ConA injection. Original magnification ×200. ***b***, Serum ALT and AST in mice injected with saline, *GFP* siRNA and *Fas* siRNA (n = 5 per group) measured 20 h after ConA injection. ***c***, Representative liver histology 1 week after 6 weekly injections of ConA in mock-treated mice and mice injected with *GFP*(sequence 1) siRNA and *Fas*(sequence 1) siRNA (n = 3 per group). Original magnification ×100. Livers of mice treated with *Fas* siRNA mice did not develop bridging fibrosis. ***d***, At 1 week after the last ConA injection in the chronic hepatitis model, hepatic hydroxyproline and serum procollagen type III (PIIINP), indicators of ongoing fibrosis, were normal in mice injected with *Fas* siRNA (n = 3 per group) but elevated in mock-treated mice and those treated with *GFP* siRNA . #, $P < 0.05$; Φ, $P < 0.01$, as compared with control groups. ***e***, Survival advantage of mice injected with *Fas* siRNA as compared to saline or *GFP* siRNA after challenge by intraperitoneal injection with Fas-specific antibody and observation for 10 d before sacrifice. RPA analysis at left (representative data from 2 independent experiments) shows specific silencing of Fas expression in hepatocytes of mice treated with *Fas* siRNA sequences 1, 5, and 6 and partial silencing with Fas sequence 2. GFP1, *GFP*(sequence 1); GFP2, *GFP*(sequence 2). The *Fas*/*Gapdh* signal ratio has been normalized to that of mock-treated mice. Sequences that silenced *Fas* by ≥80% protected against fulminant hepatitis, whereas sequences that did not silence or silenced inefficiently provided no protection. *, $P < 0.0001$. ■, GFP (dT) (n = 10); ▲, GFP (CU) (n = 15); ▼, saline (n = 15); ◇, *Fas*(sequence 1) (n = 20); ●, *Fas*(sequence 2) (n = 10); □, *Fas*(sequence 4) (n = 10); △, *Fas*(sequence 5) (n = 10); ▽, *Fas*(sequence 6) (n = 10).

expression in the liver after duplex siRNA injection protects hepatocytes from Fas-mediated apoptosis, hepatocytes from mice treated with *Fas*(sequence 1) siRNA and mock-injected mice were challenged *in vitro* with an agonistic Fas-specific antibody (Jo2) or activated hepatic mononuclear cells harvested from ConA-treated mice. *In vitro* exposure of hepatocytes from untreated or mock-treated mice to Jo2 (500 ng/ml) for 24 h resulted in 87.8 ± 6.8% (n = 5) apoptotic cells as measured by fluorescein isothiocyanate–terminal deoxynucleotide transferase-mediated dUTP nick end labeling (FITC-TUNEL) staining (Fig. 2*a*). In contrast, only 7.9 ± 1.5% (n = 5) of cultured hepatocytes from mice treated with *Fas*(sequence 1) siRNA were stained by TUNEL ($P <$ 0.0001). Moreover, protection from *in vitro* Fas-induced apoptosis after tail vein injection of other *Fas* siRNAs correlated with Fas suppression: *Fas* siRNA that did not silence had no effect on apoptosis, whereas partial silencing provided partial protection (data not shown). A recent study shows that FasL-expressing natural killer T cells are the hepatic mononuclear cells that induce hepatic cell injury in ConA-induced hepatitis[22,23]. We therefore tested, using an alanine aminotransferase (ALT) release assay, whether hepatocytes from mice treated with *Fas*(sequence 1) siRNA were resistant to cytolysis by hepatic mononuclear cells isolated from ConA-treated mice (Fig. 2*b*). Hepatocytes from mice treated with *Fas* siRNA were not lysed by hepatic mononuclear cells, whereas hepatocytes from mock-treated or untreated controls were. Thus, *Fas* silencing effectively inhibits hepatocyte apoptosis *in vitro*.

We next examined whether *Fas* siRNA treatment protects mice from fulminant hepatitis in two models of Fas-mediated liver damage. Mice treated with *Fas*(sequence 1) siRNA, *GFP*(sequence 1) siRNA or saline were challenged 1 day later by intravenous injection of ConA. Serum transaminase concentrations and liver

pathology were analyzed 20 h after ConA challenge. All control saline-treated mice and mice treated with *GFP* siRNA had extensive liver damage, showing confluent hepatocyte necrosis with bridging and inflammatory cell infiltrates surrounding the portal and central veins (Fig. 3*a*). Most surviving hepatocytes had cytoplasmic swelling, and there was frequent nuclear chromatin condensation, indicative of apoptosis. In contrast, pretreatment with *Fas* siRNA prevented liver cell necrosis and abrogated inflammatory infiltration, although mild hepatocyte swelling occurred. In ConA-induced hepatitis, release of the transaminases ALT and aspartate aminotransferase (AST) from damaged hepatocytes peaks in the serum 20 h after injection and is a good indicator of the extent of liver damage[24]. In agreement with the morphological findings, *Fas* siRNA treatment almost completely prevented the elevation of serum ALT (142 ± 58 IU/l versus 2150 ± 312 IU/l in saline-treated controls; $n = 5$, $P < 0.001$; normal, 30 IU/l) and AST (270 ± 90 IU/l versus 4120 ± 876 IU/l in saline-treated controls; $n = 5$, $P < 0.001$; normal, 50 IU/l) (Fig. 3*b*).

Fas-mediated hepatocyte apoptosis also contributes to the development of liver fibrosis in chronic hepatitis[12,25]. To evaluate further the therapeutic potential of *Fas* siRNA to treat chronic liver injury, and to determine whether siRNA administered after the noxious insult can protect in a more clinically relevant scenario, we delayed siRNA treatment until 24 h after the second of six weekly injections with a reduced dose of ConA. The siRNA injection was repeated once two weeks later. Mice were killed at seven weeks, one week after the last ConA injection. All mock-treated mice and mice treated with *GFP* siRNA developed bridging fibrosis in the liver parenchyma, whereas no hepatic fibrosis or necrosis was seen in mice treated with *Fas*(sequence 1) siRNA (Fig. 3*c*). *Fas* siRNA treatment also significantly reduced two chemical indicators of active fibrosis, hepatic hydroxyproline[26] (0.56 ± 0.17 mmol/g liver tissue in mice treated with *Fas* siRNA versus 2.13 ± 0.95 mmol/g in mock-treated controls; $n = 3$, $P < 0.05$; normal, 0.5 mmol/g) and serum procollagen type III (PIIINP)[27] (5.6 ± 1.5 ng/l in mice treated with *Fas* siRNA versus 39.2 ± 2.1 ng/l in saline-treated controls; $n = 3$, $P < 0.01$; normal 5 ng/l) (Fig. 3*d*). In addition, there was no evidence of toxicity, including lymphoproliferation, splenomegaly or other organ damage, from prolonged silencing of *Fas*, even with repeated injection (data not shown). These results indicate that treatment with *Fas* siRNA might provide protection even after the initiation of chronic liver injury.

To evaluate further whether *Fas* siRNA promotes survival in fulminant hepatitis, we challenged mice in a more aggressive hepatitis model[10] by intraperitoneal injection of a Fas-specific antibody. All control mice ($n = 40$) died within 3 days, mostly within 24 h after antibody injection. Mice treated with *Fas* siRNAs(sequences 2 or 4), which silenced expression by only 38% or not at all, also were not protected. However, mice pretreated with *Fas* siRNAs that silenced expression by 81–86% (sequences 1, 5 or 6) were protected from lethal challenge: 33 of 40 mice survived for the 10 days of observation (log-rank test, $P < 0.0001$) (Fig. 3*e*). Fatalities in the *Fas* siRNA–treated group were from hemorrhage secondary to liver failure. Liver damage in lethal fulminant hepatitis culminates within the first few weeks, but the survivors recover thereafter[28]. In fact, livers and other organs from the surviving mice appeared normal when the animals were killed at the end of the observation period (data not shown). Hence, *Fas* silencing during the acute insult prevents death from fulminant hepatitis.

Based on the crucial role that Fas-mediated apoptosis plays in a broad spectrum of immune-related liver diseases, siRNA-directed *Fas* silencing may be of therapeutic value for preventing and treating acute and chronic liver injury induced by viral and autoimmune hepatitis[2,9], alcoholic liver disease, acute and chronic liver failure[20,25] and rejection of liver transplants[2]. In fact, preliminary studies in a mouse transplant model indicate that prophylactic administration of siRNA might prevent rejection (J.W., N.O., J.M. and J.C., unpublished data). Protection from other hepatotoxic agents needs to be investigated. Extension of these results to prevent or treat other diseases, however, may require other strategies, such as viral vectors, to target other cell types and tissues. In addition, it is not clear whether hydrodynamic injection can be adapted to primates, as the volume injected into mice is a substantial fraction of the circulating blood volume. However, regional delivery of high concentrations of siRNA via hepatic artery or portal vein cannulation remains a viable alternative.

Methods

Preparation of siRNAs. siRNAs were synthesized using 2′-*O*-ACE-RNA phosphoramidites (Dharmacon Research, Lafayette, Colorado). The sense and anti-sense strands of siRNAs were: *Fas*(sequence 1), beginning at nt 364, 5′- P.GUGCAAGUGCAAACCAGACdTdT-3′ (sense), 5′- P.GUCUGGU UUGCACUUGCACdTdT-3′ (antisense); *Fas*(sequence 2), beginning at nt 874, 5′-P.AGCCGAAUGUCGCAGAACCdTdT-3′ (sense); 5′-P.GGUUCUGC-GACAUU CGGCUdTdT-3′ (antisense); *Fas*(sequence 3), beginning at nt 137, 5′-P.GGAUUAUAUCAAGGAGGCCdTdT-3′ (sense); 5′-P.GGCCUCCUU GAUAUAAUCCdTdT-3′ (antisense); *Fas*(sequence 4), beginning at nt 501, 5′-P.AUCGCCUAUGGUUGUUGACdTdT-3′ (sense); 5′-PGUCAACAACCAUA GGCGAUdTdT-3′ (antisense); *Fas*(sequence 5), beginning at nt 667, 5′-P.A UACAUCCCGAGAAUUGCUdTdT-3′ (sense); 5′-P.AGCAAUUCUCG GGAU GUAUdTdT-3′ (antisense); *Fas*(sequence 6), beginning at nt 873, 5′-P.AAG CCGAAUGUCGCAGAACdTdT-3′ (sense); 5′-P.GUUCUGCGACAUUCGGC UUdTdT (antisense). *GFP*(sequence 1), 5′- P.GGCUACGUCCAGGAGCG-CACC-3′ (sense), 5′- P.UGCGCUCCUGGACGUAGCCUU-3′ (antisense); *GFP*(sequence 2), 5′-P.GGCUACGUCCAGGAGCGCAdTdT-3′ (sense), 5′-P.U GCGCUCCUGGACGUAGCCdTdT-3′ (antisense); P represents 5′ phosphate.

RNAs were deprotected and annealed according to the manufacturer's instruction. Cy5-labeled *Fas* siRNA with the fluorophore coupled to the 3′ end of the sense strand was produced by Dharmacon Research.

siRNA treatment. Male BALB/c mice, 8–10 weeks of age and weighing 20–25 g, were purchased from the Jackson Laboratory (Bar Harbor, Maine). Synthetic siRNAs were delivered *in vivo* using a modified 'hydrodynamic transfection method'[13], by which 50 μg siRNA dissolved in 1 ml PBS was rapidly injected into the tail vein. The injection was repeated 8 and 24 h later. Control mice were injected with an equal volume of normal saline or *GFP* siRNA.

Isolation of hepatocytes. Hepatocytes were isolated by a modified hepatic portal perfusion technique[29]. The purity of hepatocytes, determined by flow cytometric analysis of intracellular albumin staining using fluorescein-conjugated goat antibody against mouse albumin (Bethyl Laboratories, Montgomery, Texas), was >90% (data not shown). For some experiments, cells were briefly cultured after plating at 2×10^6 cells per 60 mm collagen-coated culture dish in William's Medium E (Gibco-BRL, Grand Island, New York) supplemented with 10% fetal bovine serum, 15 mmol/l HEPES (pH 7.4), 1 μmol/l insulin, 2 mmol/l l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

RPA. Total RNA was extracted from hepatocytes using Trizol reagent (Molecular Research Center, Cincinnati, Ohio), and an RPA was carried out using 15 μg of total RNA and the In-vitro Transcription Kit and mouse mAPO-3 multi-probe template set (BD Pharmingen, San Diego, California) according to the manufacturer's instructions. Intensities of the protected bands were quantified by phosphorimaging (Fuji-BAS 1500, Fuji, Tokyo, Japan) based on the ratios of the mRNAs of interest to *Gapdh* (internal control).

Immunoblot. Protein extracts of mouse hepatocytes were resolved over 12% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, probed

with rabbit polyclonal antibodies against mouse Fas and then with peroxidase-conjugated goat antibodies against rabbit as the secondary antibody (Oncogene Research Product, Boston, Massachusetts), and then visualized by chemiluminescence (Amersham Life Science, Arlington Heights, Illinois).

Apoptosis assay. Primary hepatocytes from untreated mice or mice treated with *Fas* siRNA or saline were seeded in 12-well plates at a density of 1×10^5/ml. The next day Jo2 monoclonal antibody (500 ng/ml; BD Pharmingen) was added; 24 h later, liver cell apoptosis was evaluated by FITC-labeled TUNEL assay (Boehringer Mannheim, Mannheim, Germany), analyzed by flow cytometry on a FACScan flow cytometer with LYSIS II software (Nippon Becton Dickinson, Tokyo, Japan).

ALT release assay. Target liver cells, plated in 12-well plates at 1×10^4 cells/well, were co-cultivated overnight with mononuclear cells isolated from livers of mice treated with ConA (15 mg per kg body weight) at different effector-to-target ratios. Release of ALT from hepatocytes was measured in the supernatants using an ALT assay kit (Boehringer Mannheim). Cytotoxicity was expressed as the percentage of ALT in the supernatants, compared to total ALT in detergent-lysed cells.

Induction of acute hepatitis. Mice were injected intravenously through the tail vein with ConA (15 mg/kg; Sigma, St. Louis, Missouri) reconstituted in pyrogen-free saline[23]. After 20 h, serum ALT and AST were measured using a standard clinical automatic analyzer (Hitachi, type 7150, Tokyo, Japan), and paraffin-embedded liver sections were stained with H&E. Other mice were injected intraperitoneally with 8 μg of Jo2 monoclonal antibody and followed for 10 d, then killed and their livers subjected to histological examination.

Induction of liver fibrosis. A reduced dose of ConA (8 mg/kg) dissolved in pyrogen-free saline was injected in the tail vein weekly for 6 consecutive weeks[30]. Mice were treated with three injections of siRNA as above beginning 24 h after the second and fourth Con A injections and sacrificed 1 week after the final injection of ConA. Liver fibrosis was evaluated by H&E staining and measurement of hepatic hydroxyproline[26] and serum procollagen type III using a sequential saturation radioimmunoassay (Calbiochem, La Jolla, California), as described[27].

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Competing interests statement

The authors declare that they have no competing financial interests.

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1. Hannon, G.J. RNA interference. *Nature* **418**, 244–251 (2002).
2. Rust, C. & Gores, G.J. Apoptosis and liver disease. *Am. J. Med.* **108**, 567–574 (2000).
3. Elbashir, S.M. *et al.* Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–498 (2001).
4. Caplen, N.J., Parrish, S., Imani, F., Fire, A. & Morgan, R.A. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc. Natl. Acad. Sci. USA* **98**, 9742–9747 (2001).
5. McCaffrey, A.P. *et al.* RNA interference in adult mice. *Nature* **418**, 38–39 (2002).
6. Lewis, D.L., Hagstrom, J.E., Loomis, A.G., Wolff, J.A. & Herweijer, H. Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nat. Genet.* **32**, 107–108 (2002).
7. Xia, H., Mao, Q., Paulson, H.L. & Davidson, B.L. siRNA-mediated gene silencing *in vitro* and *in vivo*. *Nat. Biotechnol.* **20**, 1006–1010 (2002).
8. Kondo, T., Suda, T., Fukuyama, H., Adachi, M. & Nagata, S. Essential roles of the Fas ligand in the development of hepatitis. *Nat. Med.* **3**, 409–413 (1997).
9. Siegel, R.M. & Fleisher, T.A. The role of Fas and related death receptors in autoimmune and other disease states. *J. Allergy Clin. Immunol.* **103**, 729–738 (1999).
10. Ogasawara, J. *et al.* Lethal effect of the anti-Fas antibody in mice. *Nature* **364**, 806–809 (1993).
11. Li, X.K. *et al.* Fulminant hepatitis by Fas-ligand expression in MRL-lpr/lpr mice grafted with Fas-positive livers and wild-type mice with Fas-mutant livers. *Transplantation* **71**, 503–508 (2001).
12. Canbay, A. *et al.* Fas enhances fibrogenesis in the bile duct ligated mouse: a link between apoptosis and fibrosis. *Gastroenterology* **123**, 1323–1330 (2002).
13. Zhang, G., Budker, V. & Wolff, J.A. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum. Gene Ther.* **10**, 1735–1737 (1999).
14. Zhang, H. *et al.* Reduction of liver Fas expression by an antisense oligonucleotide protects mice from fulminant hepatitis. *Nat. Biotechnol.* **18**, 862–867 (2000).
15. Bertrand, J. *et al.* Comparison of antisense oligonucleotides and siRNAs in cell culture and *in vivo*. *Biochem. Biophys. Res. Commun.* **296**, 1000– 1004 (2002).
16. Schwarz, D.S., Hutvagner, G., Haley, B. & Zamore, P.D. Evidence that siRNAs function as guides, not primers, in the Drosophila and human RNAi pathways. *Mol. Cell* **10**, 537–548 (2002).
17. Ketting, R.F. *et al.* Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. *Genes Dev.* **15**, 2654–2659 (2001).
18. Lipardi, C., Wei, Q. & Paterson, B.M. RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. *Cell* **107**, 297–307 (2001).
19. Takahashi, T. *et al.* Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell* **76**, 969–976 (1994).
20. Ryo, K. *et al.* Significance of Fas antigen-mediated apoptosis in human fulminant hepatic failure. *Am. J. Gastroenterol.* **95**, 2047–2055. (2000).
21. Faouzi, S. *et al.* Anti-Fas induces hepatic chemokines and promotes inflammation by an NF-κB-independent, caspase-3-dependent pathway. *J. Biol. Chem.* **276**, 49077–49082 (2001).
22. Seino, K. *et al.* Contribution of Fas ligand to T cell-mediated hepatic injury in mice. *Gastroenterology* **113**, 1315–1322 (1997).
23. Takeda, K. *et al.* Critical contribution of liver natural killer T cells to a murine model of hepatitis. *Proc. Natl. Acad. Sci. USA* **97**, 5498–5503 (2000).
24. Miyazawa, Y., Tsutsui, H., Mizuhara, H., Fujiwara, H. & Kaneda, K. Involvement of intrasinusoidal hemostasis in the development of concanavalin A-induced hepatic injury in mice. *Hepatology* **27**, 497–506 (1998).
25. Galle, P.R. *et al.* Involvement of the CD95 (APO-1/Fas) receptor and ligand in liver damage. *J. Exp. Med.* **182**, 1223–1230 (1995).
26. Takahashi, S. & Lee, M.J. Collagen metabolism in fibrotic liver. Effects of concanavalin A and aggregated myeloma immunoglobin G. *Biochem. J.* **241**, 49–54 (1987).
27. Badawy, A.A. *et al.* Evaluation of colchicine with or without praziquantel therapy in the control of hepatic fibrosis in murine schistosomiasis. *Pharmacol. Res.* **33**, 319–325 (1996).
28. Dhiman, R.K., Seth, A.K., Jain, S., Chawla, Y.K. & Dilawari, J.B. Prognostic evaluation of early indicators in fulminant hepatic failure by multivariate analysis. *Dig. Dis. Sci.* **43**, 1311–1316 (1998).
29. Klauning, J.E. *et al.* Mouse liver cell culture. 1. Hepatocyte isolation. *In Vitro* **17**, 913–925 (1981).
30. Kimura, K. *et al.* Immunopathogenesis of hepatic fibrosis in chronic liver injury induced by repeatedly administered concanavalin A. *Int. Immunol.* **11**, 1491–1500 (1999).

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Potent and Specific Inhibition of Human Immunodeficiency Virus Type 1 Replication by RNA Interference

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Synthetic small interfering RNAs (siRNAs) have been shown to induce the degradation of specific mRNA targets in human cells by inducing RNA interference (RNAi). Here, we demonstrate that siRNA duplexes targeted against the essential Tat and Rev regulatory proteins encoded by human immunodeficiency virus type 1 (HIV-1) can specifically block Tat and Rev expression and function. More importantly, we show that these same siRNAs can effectively inhibit HIV-1 gene expression and replication in cell cultures, including those of human T-cell lines and primary lymphocytes. These observations demonstrate that RNAi can effectively block virus replication in human cells and raise the possibility that RNAi could provide an important innate protective response, particularly against viruses that express double-stranded RNAs as part of their replication cycle.

The phenomenon now generally termed RNA interference (RNAi) is an evolutionarily conserved process whereby the expression or introduction of double-stranded RNA (dsRNA) sequences results in the specific posttranscriptional inactivation of genes which are complementary to the dsRNA sequence used (reviewed in reference 37). In several organisms, including plants, nematodes, and fruit flies, RNAi can be used to inactivate a specific host gene subsequent to microinjection or transfection of the appropriate dsRNA (16, 21, 39). However, in mammalian cells, dsRNAs consisting of more than 30 nucleotides (nt) induce not only RNAi but also a set of responses, including activation of the interferon response, that collectively result in a global, nonspecific inhibition of host cell mRNA expression (32, 36).

Efforts to resolve the mechanisms underlying RNAi in the plant and drosophila systems revealed that longer dsRNAs were processed by a host RNase, termed dicer, into ~21-nt dsRNAs, called small interfering RNAs (siRNAs), that contain 2-nt 3′ overhangs (14, 19–21, 25, 26, 28, 43). These siRNAs are key mediators of RNAi and are thought to serve as guide RNAs for a large protein complex, termed the RNA-induced silencing complex, that is essential for target mRNA degradation (22). Importantly, it soon became apparent that direct introduction of synthetic siRNAs could also induce RNAi not only in drosophila cells but also in human cells (8, 14, 15). Subsequently, transfection of synthetic siRNAs has been used to selectively block the expression of a small but rapidly growing list of human genes and to determine the phenotypic consequences of this inhibition (1, 11, 18, 23, 25, 33).

RNAi is believed to have evolved as a host defense mechanism directed at transposable elements and infecting viruses (12, 27, 34, 38). We therefore wondered if RNAi might provide an effective defense against a pathogenic human virus. Here, we describe siRNAs targeted against the essential human immunodeficiency virus type 1 (HIV-1) regulatory proteins Tat and Rev (13) and show that these siRNAs can effectively and specifically block Tat or Rev expression and function in transfected 293T cells. More importantly, we show that these same siRNAs can prevent the productive infection of both immortalized and primary human T cells by HIV-1 in culture. These data provide evidence in favor of the hypothesis that RNAi can be used to selectively block viral gene expression, and hence replication, in human cells.

MATERIALS AND METHODS

Plasmid constructs. The mammalian expression plasmids pcTat, pcRev, pBC12/CMV, pBC12/CMV/β-Gal, pcRex, and pbTat (5, 31) and the indicator constructs pDM128/PL, pDM128/RRE, pDM128/RxRE, pTAR/CAT, and pbTAR/CAT (5, 24, 40) have been described in detail elsewhere. In addition, the HIV-1 proviral clones pNL-ADA and pNL-luc-ADA and plasmids expressing CD4 (pCMV5/CD4) and CCR5 (pCMV/CCR5) have also been described (41).

The plasmid pHIV/Tat, expressing the Tat protein under the control of the HIV-1 long terminal repeat (LTR) was constructed by PCR amplification of the *tat* cDNA sequence contained within pcTat (40). Oligonucleotide primers were designed to introduce *Hin*dIII and *Bam*HI sites so that the amplified Tat sequence could be ligated into *Hin*dIII–*Bam*HI-digested pHIV/TAR/CAT (40).

RNA interference. Twenty-one nucleotide RNA duplexes with 2-nt (2′-deoxy)-thymidine 3′ overhangs directed against nucleotides 5886 to 5909 and 8462 to 8485 of the respective HIV-1 *tat* and *rev* coding sequences were obtained from Dharmacon Research, Inc. (Lafayette, Colo.). RNAi in 293T cells was performed essentially as described previously (14). Briefly, 2×10^5 cells were plated in a 24-well plate 1 day prior to transfection. Annealed siRNA duplexes (0.1 μM) and the mammalian expression and indicator constructs denoted in the relevant figure legends were cotransfected with Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions. Tat and Rev expression was assayed by Western analysis and by biological activity as previously described (4, 31, 40).

For Jurkat T cells, annealed Tat and Rev siRNA duplexes (12 μM concentration) were mixed with 1.5×10^7 cells in 0.5 ml of OPTI-Mem I reduced serum medium (Invitrogen) and subjected to electroporation in a 0.4-cm-gap cuvette at 300 mV and 960 μF with a Bio-Rad gene pulser. The electroporated cells were immediately transferred to 15 ml of RPMI 1640–10% fetal calf serum and grown overnight in the absence of antibiotics. Inhibition of viral replication was assayed by enzyme-linked immunosorbent assay (ELISA) for p24 Gag protein production, as described below.

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Peripheral blood mononuclear cells (PBMC) from healthy HIV-1-negative donors were isolated by Ficoll-Hypaque gradient centrifugation. Cells, 10^6/ml, were frozen at −150°C in human AB serum until required. Thawed cells were resuspended in AIM V medium (Invitrogen) and supplemented with 10% human AB serum (Sigma) and 10 U of interleukin-2 (R&D Systems) per ml as described previously (41). Nonadherent cells were stimulated with 3 μg of phytohemagglutinin (Sigma) per ml for 2 days prior to electroporation as described above.

Cell maintenance and preparation of viral stocks. Human 293T cells were maintained as described previously (4), while Jurkat T cells were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum. Fresh viral stocks were prepared by transfection of 293T cells with 1 μg of pNL-ADA or pNL-luc-ADA (41) with FuGENE 6 reagent (Roche). After 48 h, the cells were washed once with phosphate-buffered saline (PBS), and newly produced virions were harvested over 3 h in 0.5 ml of fresh medium. Viral supernatants were clarified by passage through a 0.4-μm syringe filter. HIV-1 stocks pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G) were prepared in a similar manner, except that 100 ng of pHIT/G (17) was cotransfected with 1 μg of pNL-ADA with FuGENE 6 reagent (Roche).

Viral replication assays. 293T cells were cotransfected with 100 ng of pCMV5/CD4, 500 ng of pCMV/CCR5, and siRNA oligonucleotides, as denoted in the relevant figure legends. After 48 h, $CD4^+$ $CCR5^+$ 293T cells were infected with 50 ng of p24 antigen of the NL-ADA or NL-luc-ADA virus (41). The following day, the cells were washed extensively with PBS and overlaid with fresh growth medium. Supernatants were harvested 48 h later and p24 Gag antigen production was quantified by ELISA (NEN). In the case of Jurkat T cells, cells were harvested 24 h after electroporation and counted. Cells (10^6) were then infected with 50 ng of p24 antigen of VSV-G-pseudotyped NL-ADA virus in a 24-well dish as described above. Progeny virus production was measured 48 h later by p24 Gag ELISA. Similarly, PBMC were also infected 24 h after electroporation with 50 ng of p24 of the CCR5-tropic virus NL-ADA, and progeny virus production was measured 48 h later.

Viral gene expression was measured in 293T cells with a luciferase reporter virus (10). After 48 h, the cells were washed with PBS and resuspended in 200 μl of cell lysis buffer (Promega) and luciferase activity was determined as previously described (41).

RNA, DNA, and Western analyses. All nucleic acid blotting and hybridization techniques were performed essentially as described previously (35). Total RNA was isolated from infected 293T cells with a QIAGEN RNeasy minikit in accordance with the manufacturers' instructions. RNA (15 μg) was separated on a 1% agarose gel containing formaldehyde and transferred to a Hybond-N membrane (Amersham Pharmacia Biotech). RNA was fixed by UV cross-linking in a Stratalinker (Stratagene), and HIV-1 RNAs were detected with a ^{32}P-labeled random-primed DNA probe derived from pNL-ADA by PCR amplification with oligonucleotide primers directed towards the HIV-1 U3 region (nucleotides 9078 to 9609).

Total DNA was extracted from infected 293T cells with a QIAGEN DNeasy tissue kit in accordance with the manufacturers' instructions. Samples were treated with 400 μg of RNase A to remove contaminating RNA. DNA (10 μg) was dried in a speed vacuum, denatured in 2 μl of 0.2 M NaOH for 15 min at 37°C, and spotted onto a Hybond-N membrane. After UV cross-linking, the membrane was probed with the HIV-1-specific ^{32}P-labeled probe as described above or with a random-primed β-globin cDNA probe.

Western analysis of protein expression in transfected 293T cells was performed with rabbit polyclonal antisera directed against the HIV-1 Tat or Rev protein or against human Tap/NXF1, as previously described (9, 31). All antibodies were used at a dilution of 1:1,000.

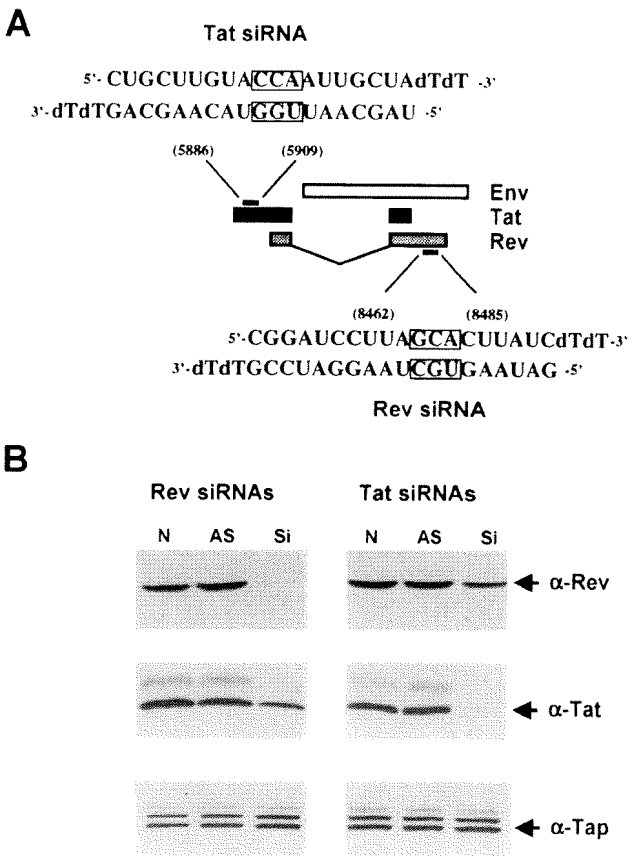

FIG. 1. (A) Schematic representation of part of the HIV-1 genome. HIV-1 *tat* and *rev* exons are depicted with black and grey boxes, respectively, while the *env* open reading frame is shown as a white box (not to scale). The structure and target coordinates of the siRNAs specific for *tat* and *rev* are depicted. Boxed residues were mutated by transversion for use as control duplexes in some experiments. (B) siRNAs directed against *tat* and *rev* specifically inhibit Tat and Rev protein expression. Human 293T cells were cotransfected with 50 ng of both the pcTat and pcRev expression plasmid in the absence of RNA oligonucleotides (N) or in the presence of 0.1 μM antisense oligonucleotides (AS) or 0.1 μM siRNA duplexes (Si). Cell lysates were prepared 60 h posttransfection, and protein expression was assayed by Western analysis with polyclonal antiTat and antiRev antisera. Western blots were also probed with antibodies directed against the endogenous TAP/NXF-1 protein, which here served as an internal control for both loading and specificity.

RESULTS

In the context of the HIV-1 genome, the viral Tat and Rev proteins are encoded by two partially overlapping exons separated by an intron consisting largely of envelope coding sequences (Fig. 1A). Previously, Elbashir et al. (14) demonstrated that synthetic 21-nt siRNA duplexes, bearing 2-nt 3′ overhangs, can function as effective inhibitors of mRNA expression in transfected human cells. In addition, these researchers suggested that the 2-nt overhang should ideally consist of uridine or, in the interest of stability, deoxythymidine. Based on these observations, we therefore searched the nonoverlapping parts of the Tat and Rev coding sequences for appropriate siRNA targets. The location of the sequences in the HIV-1 Tat and Rev open reading frame that were chosen as targets and the structure of the synthetic siRNAs that were used in this analysis are shown in Fig. 1A.

Specific inhibition of HIV-1 Tat and Rev expression and function by RNAi. To test whether siRNAs that were targeted to these short Tat and Rev sequence elements would specifically block Tat or Rev protein expression, we cotransfected 293T cells with the Tat expression construct pcTat and the Rev expression plasmid pcRev (31). These cDNA expression plasmids contain exclusively the coding sequence of Tat or Rev (Fig. 1A), and therefore each should be subject to inhibition by only one of the siRNAs. Cells were also cotransfected with one

of the double-stranded siRNAs or, as controls, with only the relevant antisense RNA strand or with nothing.

As shown in Fig. 1B, Western analysis revealed that the Tat-specific siRNA blocked Tat protein expression but did not significantly affect expression of an irrelevant cellular protein termed Tap. Similarly, the Rev-specific siRNA also selectively blocked expression of the Rev protein. In neither case did the antisense RNA oligonucleotide alone exert any effect. In some experiments, we did notice a slight (≤20%) effect of the Rev-specific siRNA on Tat protein expression or of the Tat-specific siRNA on Rev expression. This may reflect a weak nonspecific inhibitory effect or, more trivially, may suggest that the double-stranded siRNA, unlike the single-stranded antisense RNA, slightly reduced transfection efficiency in this experiment. Nevertheless, it is clear that these siRNAs can indeed selectively block expression of the HIV-1 Tat or Rev protein in transfected 293T cells.

We next wished to determine whether these siRNAs would also block Tat or Rev function. The HIV-1 Tat protein serves as a specific transcriptional transactivator of gene expression directed by the HIV-1 LTR promoter after binding to a specific target site termed TAR (reviewed in reference 13). As shown in Fig. 2A, we confirmed that the HIV-1 Tat protein expressed under the control of either the cytomegalovirus (CMV) immediate early promoter or the HIV-1 LTR itself can strongly activate chloramphenicol acetyltransferase (CAT) activity directed by the HIV-1 LTR promoter in transfected 293T cells. As shown in Fig. 2A, this activation was potently inhibited by cotransfection of the specific siRNA but not by cotransfection of either the sense or antisense RNA strand alone. This inhibition is specific, since activation by the bovine immunodeficiency virus (BIV) Tat protein (bTat) of an HIV-1 LTR promoter that contains the BIV TAR element in place of the HIV-1 TAR element was not affected (Fig. 2A). Importantly, while the bTat protein is very similar to HIV-1 Tat in its mechanism of action, the HIV-1 Tat coding sequence indicated in Fig. 1A is not conserved in bTat (5).

As shown in Fig. 2B, we demonstrated that the siRNA targeted to the HIV-1 Rev protein can also specifically block HIV-1 Rev function in transfected 293T cells. This experiment utilizes an indicator construct termed pDM128/RRE that contains an mRNA in which the *cat* gene and the HIV-1 Rev response element (RRE) are sequestered within an intron (4, 24). This RNA is normally unable to exit the cell nucleus but is exported and expressed in the presence of the HIV-1 Rev nuclear RNA export factor. As shown in Fig. 2B, this induction is blocked by the Rev-specific siRNA but not by either the sense or antisense RNA strand alone. This inhibition is specific, since the Rev siRNA did not inhibit expression of the unspliced *cat* mRNA encoded by the similar indicator plasmid pDM128/RxRE, which contains the human T-cell leukemia virus (HTLV) Rex response element in place of the RRE, in the presence of the functionally analogous but unrelated HTLV Rex protein (4).

Inhibition of HIV-1 gene expression and replication by RNAi. Although the human cell line 293T is not normally infectible by HIV-1, 293T cells can support HIV-1 infection and replication if they are programmed to express the CD4 receptor and the CCR5 coreceptor for HIV-1 (41). We have previously described the pNL-ADA and pNL-luc-ADA HIV-1

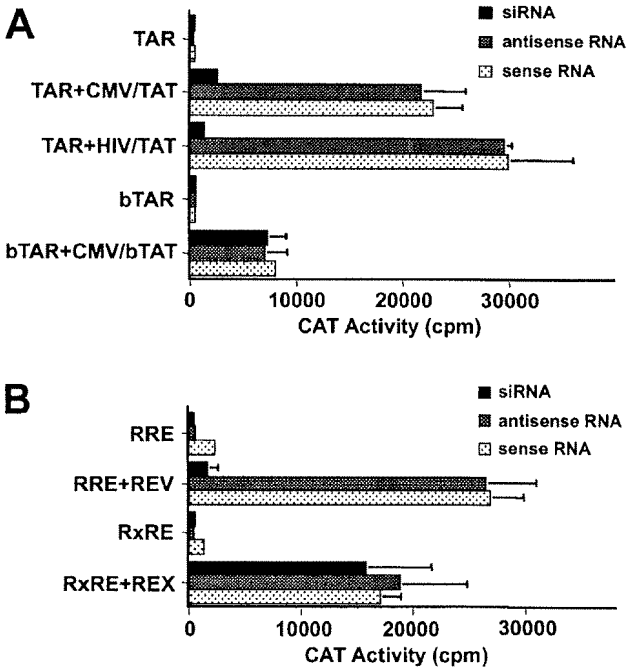

FIG. 2. (A) siRNAs directed against HIV-1 *tat* inhibit HIV-1 Tat but not BIV Tat function. Human 293T cells were transfected with 100 ng of pTAR/CAT or pbTAR/CAT, 50 ng of the internal control plasmid pBC12/CMV/β-Gal, and 0.1 ng of the indicated effector plasmid. siRNA oligonucleotides or siRNA duplexes were cotransfected with the reporter and expression plasmids as described in Materials and Methods. Cells were harvested 48 h after transfection, and induced CAT activities were determined. The data shown represent averages for three experiments with the standard deviations indicated by error bars. (B) siRNAs directed against HIV-1 *rev* specifically inhibit HIV-1 Rev but not HTLV Rex function. 293T cells were transfected with 25 ng of the pDM128/RRE or pDM128/RxRE reporter plasmid, 50 ng of pBC12/CMV/β-Gal, and 1 ng of the indicated effector plasmid. siRNA oligonucleotides or siRNA duplexes were cotransfected with the reporter and expression plasmids as described in Materials and Methods. Cells were harvested 48 h after transfection, and induced CAT and β-galactosidase activities were determined. The data shown represent averages for three experiments with the standard deviations indicated by error bars.

proviral expression plasmids. The NL-ADA virus is a fully replication-competent HIV-1 that was derived from the NL4-3 HIV-1 isolate by substitution of the NL4-3 *env* gene with an *env* gene derived from the CCR5-tropic HIV-1 isolate ADA (41, 42). NL-luc-ADA is similar to NL-ADA except that the HIV-1 *nef* gene has been replaced by the *luc* indicator gene (10). Therefore, infection of cells by NL-luc-ADA can be readily monitored by measurement of the level of luciferase expression.

We initially determined the effect of the siRNAs on the level of viral gene expression by infecting 293T cells transfected with plasmids encoding CD4 and CCR5, as well as with RNA oligonucleotides, with the NL-luc-ADA indicator virus. As shown in Fig. 3A, both the Tat siRNA and the Rev siRNA, and particularly the combination of both, were able to effectively inhibit expression of the virally encoded *luc* indicator gene in these HIV-1-infected cells. In contrast, the antisense RNA oligonucleotides alone had no specific effect.

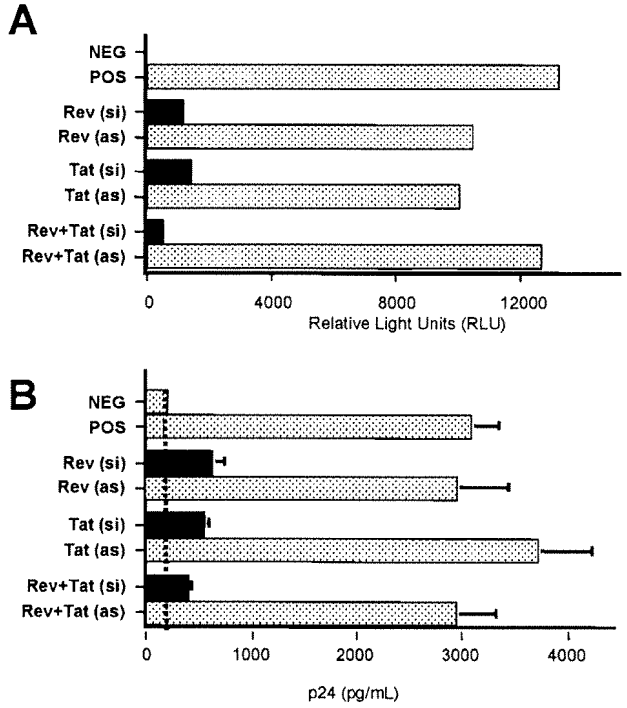

FIG. 3. Inhibition of HIV-1 replication in human cells by RNAi. 293T cells were transfected with CD4 and CCR5 expression plasmids and siRNA duplexes (si) or antisense RNAs (as) (0.1 μM each). After 48 h, $CD4^+$ $CCR5^+$ cells were infected overnight with 50 ng of p24 antigen of the luciferase reporter virus NL-luc-ADA or the wild-type virus NL-ADA. Viral gene expression was determined by luciferase activity after 48 h of infection (A), while viral replication was measured by p24 Gag antigen production 60 h posttransfection (B). 293T cells lacking CD4 and CCR5 served as the negative control (NEG), while $CD4^+$ $CCR5^+$ 293T cells infected in the absence of siRNAs served as the positive control (POS). A low level of carryover of the virus stock used to infect the cells explains the background level of p24 Gag seen in panel B (vertical dashed line).

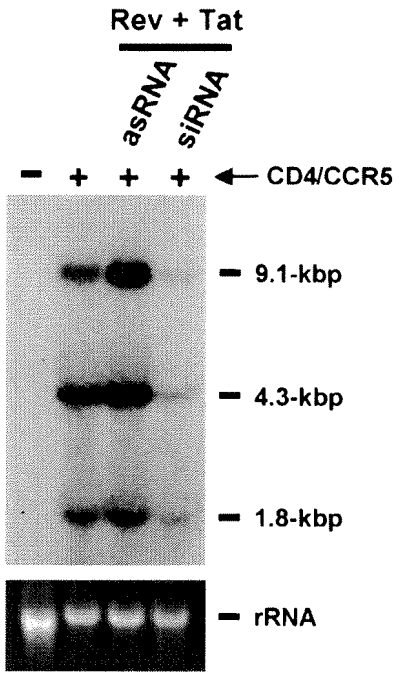

FIG. 4. Analysis of HIV-1 RNA expression in human cells reveals that *tat* and *rev* siRNAs can reduce the expression of all three classes of HIV mRNA. Human 293T cells were cotransfected with siRNA duplexes (si) or with antisense RNAs (as) (0.1 μM each) and the CD4 and CCR5 expression plasmids as indicated at the top of the lanes. $CD4^+$ $CCR5^+$ cells were then infected overnight with 50 ng of p24 antigen. After 48 h of infection, total RNA was extracted from infected 293T cells and subjected to Northern analysis. The approximate sizes of the three classes of HIV-1 transcripts are indicated on the right. 18S rRNA, stained with ethidium bromide, is shown at the bottom of the figure as a loading control.

We next examined whether the siRNAs would be able to inhibit the release of progeny HIV-1 virion particles from infected 293T cells. As shown in Fig. 3B, the two siRNAs acting either alone or in combination were indeed able to effectively inhibit the production of progeny HIV-1 virions. Again, the antisense RNA oligonucleotides had little or no effect. We therefore conclude that these siRNAs are able to effectively inhibit HIV-1 gene expression and replication in infected $CD4^+$ $CCR5^+$ 293T cells.

RNAi is predicted to induce the selective degradation of mRNAs containing the appropriate target sequence (37), and we therefore wished to confirm that the siRNAs were indeed reducing the steady-state expression of HIV-1 mRNAs in the infected 293T cells. Although the siRNAs used were targeted to the *tat* and *rev* genes of HIV-1, which are encoded by the small, multiply spliced class of HIV-1 mRNAs, it is predicted that the siRNAs would also reduce the expression of the unspliced and singly spliced class of mRNAs contained in HIV-1. This could be achieved either directly, given that many of these mRNAs retain *tat* and *rev* sequences within their 3′ untranslated region, or indirectly, by blocking their production by interfering with Tat and/or Rev synthesis. As shown in Fig. 4, we indeed observed a marked reduction in the level of expression of all three classes of HIV-1 mRNAs in the infected $CD4^+$ $CCR5^+$ cells that had been transfected with both siRNAs relative to levels in infected cells that were transfected with only one RNA strand or cells that were mock transfected.

While the result shown in Fig. 4 is consistent with the hypothesis that the siRNAs are blocking HIV-1 gene expression and replication by inducing the degradation of viral mRNA species, it is also possible that the siRNAs could act to block infection directly by inducing the degradation of the RNA genome present in the infecting HIV-1 virion prior to reverse transcription. To examine this possibility, we quantified the level of HIV-1 proviral DNA production in HIV-1-infected $CD4^+$ $CCR5^+$ 293T cells by Southern dot blot analysis (Fig. 5). The endogenous β-globin gene was used as a loading control.

As shown in Fig. 5, we were indeed able to readily detect HIV-1 proviral DNA in HIV-1-infected $CD4^+$ $CCR5^+$ 293T cells but not in 293T cells lacking CD4 and CCR5 that were incubated with the same infectious virus dose. The level of proviral DNA production was reduced but not totally eliminated in a $CD4^+$ $CCR5^+$ culture treated with the reverse transcriptase inhibitor azidodeoxythymidine (AZT) (2), thus arguing that the bulk of the observed signal does represent newly synthesized viral DNA. Interestingly, treatment of infected cells with the siRNAs also modestly reduced the level of

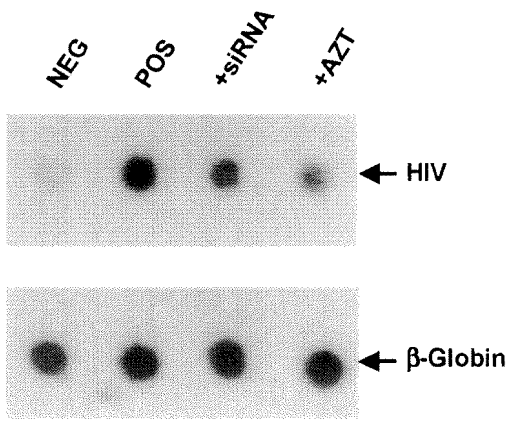

FIG. 5. siRNAs directed against *tat* and *rev* can initiate the degradation of HIV-1 genomic RNA prior to reverse transcription. Human 293T cells were transfected with siRNA duplexes (si) or with antisense RNAs (as). $CD4^+$ $CCR5^+$ cells were infected overnight with 50 ng of p24 antigen. After 48 h, total DNA was extracted from infected 293T cells, denatured in 0.2 M NaOH, spotted onto a nylon membrane, and probed with a ^{32}P-labeled HIV-1-specific probe as described in Materials and Methods. As a control for loading, equivalent amounts of DNA were probed with a ^{32}P-labeled β-globin probe. 293T cells treated with 0.1 μM AZT, the reverse transcriptase inhibitor (2), are also shown (+AZT). DNA isolated from mock-infected 293T cells served as the negative control (NEG), while $CD4^+$ $CCR5^+$ 293T cells infected in the absence of siRNAs served as the positive control (POS).

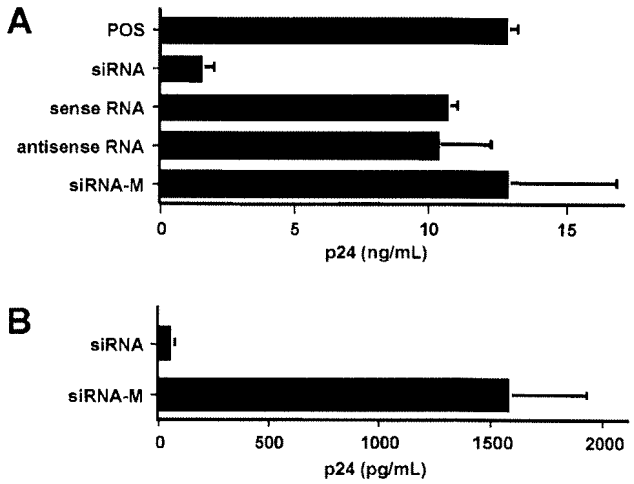

FIG. 6. siRNA inhibition of HIV-1 replication in T cells. (A) Jurkat T cells were transfected with a 12 μM concentration of each of the indicated siRNA duplex or single-stranded RNA oligonucleotides. Mock-transfected Jurkat T cells served as the positive control (POS), while the two 3-nt mismatch siRNAs shown in Fig. 1A served as the control (siRNA-M). After 24 h, transfected cells were infected overnight with VSV-G-pseudotyped NL-ADA. Viral production was determined by measurement of p24 Gag production at 48 h after infection. The data shown represent averages for three experiments with the standard deviations indicated by error bars. (B) Human PBMCs were transfected with the wild-type or mutant forms of the Tat and Rev siRNAs by electroporation and were infected with the R5-tropic NL-ADA strain 24 h later. Progeny virus production was measured 48 h after infection by p24 ELISA.

HIV-1 proviral DNA detected. While this result suggests that siRNAs can indeed inhibit HIV-1 infection to some degree, presumably by inducing the degradation of the viral RNA genome prior to completion of reverse transcription, the effect illustrated in Fig. 5 does not appear sufficient to explain the dramatic drop in viral gene expression induced by these siRNAs in HIV-1-infected $CD4^+$ $CCR5^+$ 293T cells (Fig. 3A).

Inhibition of HIV-1 replication in T cells by RNAi. All of the experiments reported thus far, including the HIV-1 infection experiments, have used human 293T cells as an experimental system. 293T cells are fully competent to support HIV-1 infection when engineered to express the appropriate receptors, and they are readily transfected with synthetic siRNAs. However, these cells may not be representative of the tissues, including $CD4^+$ T cells, that are infected by HIV-1 in vivo.

To examine whether RNAi could also inhibit HIV-1 replication in human T cells, we first determined the effect of the siRNAs characterized above on HIV-1 replication in the Jurkat human $CD4^+$ T-cell line. As shown in Fig. 6A, we indeed observed a significant inhibition in progeny virus release from HIV-1-infected Jurkat T cells that had been transfected with the wild-type Tat and Rev siRNAs relative to T cells that were transfected with either RNA strand alone or with control siRNAs bearing a 3-nt missense mutation (Fig. 1A). Importantly, introduction of such central mismatches has been previously reported to block RNAi (15). Next, we asked whether RNAi would inhibit HIV-1 replication in human peripheral blood lymphocytes. In fact, relative to mutant forms of the same siRNA duplexes, the wild-type Tat and Rev siRNAs produced a dramatic drop in the release of progeny virions from HIV-1-infected peripheral blood lymphocytes (Fig. 6B). Therefore, we conclude that RNAi is functional in cells of lymphoid origin and that RNAi can be used to effectively inhibit HIV-1 replication in human T cells in culture.

DISCUSSION

The clearance of viral infections from mammalian hosts normally requires pathogen-specific immune responses, such as neutralizing antibodies and cytotoxic T lymphocytes. However, target-nonspecific or innate defenses can also play an important protective role, particularly in the early stages of the infection prior to the clonal expansion of lymphocytes that are able to mount target-specific responses. The most important and best understood innate response to many viral infections is that mounted by the interferon system (reviewed in reference 36). This complex system is designed to block the spread of viruses within the infected host by blocking the production of progeny virions. Importantly, activation of the interferon response can also profoundly inhibit the synthesis of host cell proteins and may even result in the death of the infected cell.

One potent activator of the interferon system is dsRNA molecules that are larger than ~30 nt (32). Mammalian cells therefore differ from the cells of lower animal species, such as nematodes and fruit flies, which respond to introduced dsRNA molecules primarily by inducing RNAi (16, 21). Indeed, for a long time it remained unclear whether mammalian cells could even mount an RNAi response, given the profound physiological changes that are induced upon activation of the interferon response by longer dsRNAs (7).

The demonstration that synthetic siRNAs of ~21 nt can

induce the specific inhibition of target mRNA expression in human cells without inducing the interferon response (8, 14) has raised the issue of whether RNAi might also play an important role in antiviral defense in mammalian, and particularly human, cells, as indeed has been previously demonstrated in plant cells (12, 34). In this report, we have taken a first step towards addressing this issue by demonstrating that RNAi induced by transfection of synthetic siRNAs can indeed effectively protect human cells, including primary T cells, from the pathogenic retrovirus HIV-1 (Fig. 3 through 6). RNAi was able to potently and specifically inhibit HIV-1 gene expression and replication by inhibiting viral infection (Fig. 5) and by reducing viral mRNA expression (Fig. 4). These data therefore indicate that RNAi has the potential to protect human cells against viral infection, and to prevent virus spread, independently of the normally overlying interferon response.

Clearly, this observation raises a number of important questions, including the following. (i) Is RNAi normally activated by viruses that produce dsRNAs as part of their replication cycle? Such viruses include all RNA viruses as well as some DNA viruses, such as adenovirus. If so, then one would predict that virus-specific siRNAs might be produced in infected cells. (ii) Does induction of RNAi exert an inhibitory effect on virus replication and spread? This question could be addressed in cell culture by using synthetic siRNAs to block proteins, such as dicer, that are known to be essential for de novo siRNA production (21, 25). (iii) Have viruses evolved mechanisms to specifically inhibit RNAi, in addition to the mechanisms that many viruses have evolved to overcome the inhibitory effect of the interferon response on their replication (36)? Ample precedent for such viral defense mechanisms exists in the case of plants (reviewed in reference 29). (iv) Could RNAi be used to protect humans or animals against viral infection? The recent demonstration that biologically active siRNAs can be transcribed from DNA templates (6, 44) suggests that it might be possible to protect human or animal cells against specific virus infections by constitutively expressing siRNAs that are specific for conserved viral sequence elements, as indeed has been previously reported for plants (30). In the case of economically important animal species, it might eventually prove feasible to construct transgenic animals that constitutively express siRNAs that are targeted against important viral pathogens, a potentially powerful variation on the previously proposed concept of "intracellular immunization" (3). In effect, this could render the animal entirely nonpermissive for the targeted virus. Clearly, the potential role of RNAi in antiviral defense is an issue that merits considerable future attention.

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REFERENCES

1. **Ancellin, N., C. Colmont, J. Su, Q. Li, N. Mittereder, S. S. Chae, S. Stefansson, G. Liau, and T. Hla.**2002. Extracellular export of sphingosine kinase-1 enzyme. Sphingosine 1-phosphate generation and the induction of angiogenic vascular maturation. J. Biol. Chem. **277:**6667–6675.
2. **Arts, E. J., M. E. Quiñones-Mateu, J. L. Albright, J.-P. Marois, C. Hough, Z. Gu, and M. A. Wainberg.**1998. 3′-Azido-3′-deoxythymidine (AZT) mediates cross-resistance to nucleoside analogs in the case of AZT-resistant human immunodeficiency virus type 1 variants. J. Virol. **72:**4858–4865.
3. **Baltimore, D.**1988. Intracellular immunization. Nature **335:**395–396.
4. **Bogerd, H. P., A. Echarri, T. M. Ross, and B. R. Cullen.**1998. Inhibition of human immunodeficiency virus Rev and human T-cell leukemia virus Rex function, but not Mason-Pfizer monkey virus constitutive transport element activity, by a mutant human nucleoporin targeted to Crm1. J. Virol. **72:**8627–8635.
5. **Bogerd, H. P., H. L. Wiegand, P. D. Bieniasz, and B. R. Cullen.**2000. Functional differences between human and bovine immunodeficiency virus Tat transcription factors. J. Virol. **74:**4666–4671.
6. **Brummelkamp, T. R., R. Bernards, and R. Agami.**2002. A system for stable expression of short interfering RNAs in mammalian cells. Science **296:**550–553.
7. **Caplen, N. J., J. Fleenor, A. Fire, and R. A. Morgan.**2000. dsRNA-mediated gene silencing in cultured Drosophila cells: a tissue culture model for the analysis of RNA interference. Gene **252:**95–105.
8. **Caplen, N. J., S. Parrish, F. Imani, A. Fire, and R. A. Morgan.**2001. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. Proc. Natl. Acad. Sci. USA **98:**9742–9747.
9. **Coburn, G. A., H. L. Wiegand, Y. Kang, D. N. Ho, M. M. Georgiadis, and B. R. Cullen.**2001. Using viral species specificity to define a critical protein/RNA interaction surface. Genes Dev. **15:**1194–1205.
10. **Connor, R. I., B. K. Chen, S. Choe, and N. R. Landau.**1995. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. Virology **206:**935–944.
11. **Cortez, D., S. Guntuku, J. Qin, and S. J. Elledge.**2001. ATR and ATRIP: partners in checkpoint signaling. Science **294:**1713–1716.
12. **Covey, S. N., N. S. Al-Kaff, A. Langara, and D. S. Turner.**1997. Plants combat infection by gene silencing. Nature **385:**781–782.
13. **Cullen, B. R.**1998. HIV-1 auxiliary proteins: making connections in a dying cell. Cell **93:**685–692.
14. **Elbashir, S. M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl.** 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature **411:**494–498.
15. **Elbashir, S. M., J. Martinez, A. Patkaniowska, W. Lendeckel, and T. Tuschl.**2001. Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. EMBO J. **20:**6877–6888.
16. **Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello.**1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature **391:**806–811.
17. **Fouchier, R. A. M., B. E. Meyer, J. H. M. Simon, U. Fischer, and M. H. Malim.**1997. HIV-1 infection of non-dividing cells: evidence that the amino-terminal basic region of the viral matrix protein is important for Gag processing but not for post-entry nuclear import. EMBO J. **16:**4531–4539.
18. **Garrus, J. E., U. K. von Schwedler, O. W. Pornillos, S. G. Morham, Z. H. Zavitz, H. E. Wang, D. A. Wettstein, K. M. Stray, M. Côté, R. L. Rich, D. G. Myszka, and W. I. Sundquist.**2001. Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. Cell **107:**55–65.
19. **Grishok, A., A. E. Pasquinelli, D. Conte, N. Li, S. Parrish, I. Ha, D. L. Baillie, A. Fire, G. Ruvkun, and C. C. Mello.**2001. Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell **106:**23–34.
20. **Hamilton, A. J., and D. C. Baulcombe.**1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science **286:**950–952.
21. **Hammond, S. M., E. Bernstein, D. Beach, and G. J. Hannon.**2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature **404:**293–295.
22. **Hammond, S. M., S. Boettcher, A. A. Caudy, R. Kobayashi, and G. J. Hannon.**2001. Argonaute2, a link between genetic and biochemical analyses of RNAi. Science **293:**1146–1150.
23. **Harborth, J., S. M. Elbashir, K. Bechert, T. Tuschl, and K. Webe.**2001. Identification of essential genes in cultured mammalian cells using small interfering RNAs. J. Cell Sci. **114:**4557–4565.
24. **Hope, T. J., X. Huang, D. McDonald, and T. G. Parslow.**1990. Steroid-receptor fusion of the human immunodeficiency virus type 1 Rev transactivator: mapping cryptic functions of the arginine-rich motif. Proc. Natl. Acad. Sci. USA **87:**7787–7791.
25. **Hutvágner, G., J. McLachlan, A. E. Pasquinelli, É. Bálint, T. Tuschl, and P. D. Zamore.**2001. A cellular function for the RNA-interference enzyme dicer in the maturation of the *let-7* small temporal RNA. Science **293:**834–838.
26. **Ketting, R. F., S. E. Fischer, E. Bernstein, T. Sijen, G. J. Hannon, and R. H. Plasterk.**2001. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev. **15:**2654–2659.
27. **Ketting, R. F., T. H. Haverkamp, H. G. van Luenen, and R. H. Plasterk.**1999. Mut-7 of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. Cell **99:**133–141.
28. **Knight, S. W., and B. L. Bass.**2001. A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in C. elegans. Science **293:**2269–2271.

29. **Li, W. X., and S. W. Ding.**2001. Viral suppressors of RNA silencing. Curr. Opin. Biotechnol. **12:**150–154.
30. **Lindbo, J. A., L. Silva-Rosales, W. M. Proebsting, and W. G. Dougherty.**1993. Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resistance. Plant Cell **5:**1749–1759.
31. **Malim, M. H., J. Hauber, S.-Y. Le, J. V. Maizel, and B. R. Cullen.**1989. The HIV-1 *rev trans*-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature **338:**254–257.
32. **Manche, L., S. R. Green, C. Schmedt, and M. B. Mathews.**1992. Interactions between double-stranded RNA regulators and the protein kinase DAI. Mol. Cell. Biol. **12:**5238–5248.
33. **Martins, L. M., I. Iaccarino, T. Tenev, S. Gschmeissner, N. F. Totty, N. R. Lemoine, J. Savopoulos, C. W. Gray, C. L. Creasy, C. Dingwall, and J. Downward.**2002. The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a reaper-like motif. J. Biol. Chem. **277:**439–444.
34. **Ratcliff, F., B. D. Harrison, and D. C. Baulcombe.**1997. A similarity between viral defense and gene silencing in plants. Science **276:**1558–1560.
35. **Sambrook, J., and D. W. Russell.**2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
36. **Sen, G. C.**2001. Viruses and interferons. Annu. Rev. Microbiol. **55:**255–281.
37. **Sharp, P. A.**2001. RNA interference-2001. Genes Dev. **15:**485–490.
38. **Tabara, H., M. Sarkissian, W. G. Kelly, J. Fleenor, A. Grishok, L. Timmons, A. Fire, and C. C. Mello.**1999. The *rde-1* gene, RNA interference and transposon silencing in *C. elegans*. Cell **99:**123–132.
39. **Tenllado, F., and J. R. Díaz-Ruíz.**2001. Double-stranded RNA-mediated interference with plant virus infection. J. Virol. **75:**12288–12297.
40. **Tiley, L. S., S. J. Madore, M. H. Malim, and B. R. Cullen.**1992. The VP16 transcription activation domain is functional when targeted to a promoter-proximal RNA sequence. Genes Dev. **6:**2077–2087.
41. **Tokunaga, K., M. L. Greenberg, M. A. Morse, R. I. Cumming, H. K. Lyerly, and B. R. Cullen.**2001. Molecular basis for cell tropism of CXCR4-dependent human immunodeficiency virus type 1 isolates. J. Virol. **75:**6776–6785.
42. **Westervelt, P., H. E. Gendelman, and L. Ratner.**1991. Identification of a determinant within the human immunodeficiency virus 1 surface envelope glycoprotein critical for productive infection of primary monocytes. Proc. Natl. Acad. Sci. USA **88:**3097–3101.
43. **Zamore, P. D., T. Tuschl, P. A. Sharp, and D. P. Bartel.**2000. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell **101:**25–33.
44. **Zeng, Y., E. J. Wagner, and B. R. Cullen.** 2002. Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. Mol. Cell, in press.Cell **9:**1327–1333.

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Inhibition of Human Immunodeficiency Virus Type 1 Replication in Primary Macrophages by Using Tat- or CCR5-Specific Small Interfering RNAs Expressed from a Lentivirus Vector

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Although several groups have demonstrated that RNA interference, induced by transfection of small interfering RNA (siRNA) duplexes, can protect cells against a viral challenge in culture, this protection is transient. Here, we describe lentivirus expression vectors that can stably express siRNAs at levels sufficient to block virus replication. We have used these vectors to stably express siRNAs specific for the essential human immunodeficiency virus type 1 (HIV-1) Tat transcription factor or specific for a cellular coreceptor, CCR5, that is required for infection by the majority of primary HIV-1 isolates. These lentivirus vectors are shown to protect cells, including primary macrophages, against HIV-1 infection in culture by inducing selective degradation of their target mRNA species. These data suggest that it should be possible to block the expression of specific viral or cellular genes in vivo by using viral vectors to stably express the appropriate siRNAs.

RNA interference (RNAi) was first described in nematodes as a specific mechanism of posttranscriptional gene silencing induced by introduction of long double-stranded RNA (dsRNA) molecules homologous to the target mRNA (9). During RNAi, these long dsRNA molecules are cleaved into ~21-bp dsRNAs, termed small interfering RNA (siRNA) duplexes, by a cellular enzyme called dicer (13, 16, 20, 22). One strand of this duplex is then incorporated into the RNA induced silencing complex (RISC), where it acts as a guide RNA that specifically targets RISC proteins to homologous mRNAs (13, 28, 40). Once RISC has bound an mRNA bearing a perfectly matched sequence, the mRNA is cleaved by an unknown endonuclease component. After release of the RISC, the mRNA is degraded by cellular exonucleases, thus resulting in specific posttranscriptional silencing of the target gene (14, 17, 30).

Although RNAi was first described in *Caenorhabditis elegans*, it quickly became apparent that RNAi is closely related to similar phenomena previously described in plants and certain fungi and it is now clear that the ability to mount an RNAi response is conserved among all higher eukaryotes (14, 17, 30). RNAi and related phenomena have been found to play important roles in the regulation of gene expression during development and in defense of the genome against random mutagenesis induced by transposable elements (14, 17, 21, 30, 45).

An additional activity mediated by RNAi, at least in plants, is antiviral defense (7, 37). Although it remains unclear whether RNAi also has a normal role in antiviral defense in vertebrates, the induction of an RNAi response can confer specific protection against several viral pathogens in culture, including human immunodeficiency virus type 1 (HIV-1), hepatitis C virus, influenza virus, poliovirus, respiratory syncytial virus, and hepatitis B virus (2, 5, 11, 12, 15, 18, 19, 23, 33, 36, 41, 44, 50). There has therefore been considerable interest in the development of RNAi as a possible treatment for virus-induced diseases.

Although RNAi can be effectively induced in many lower eukaryotes by the simple introduction of long dsRNAs, this is not possible in vertebrate cells because of activation of the interferon response, which causes a global inhibition of mRNA translation and frequently leads to apoptosis (27). However, a gene-specific RNAi response can be induced by direct transfection of ~21-bp siRNA duplexes or, alternatively, by introduction of plasmids that encode short RNA hairpins that can be processed by dicer to give siRNA duplexes (3, 8, 34, 43, 51). Because induction of the interferon response requires dsRNAs of >30 bp (27), this strategy allows specific gene silencing by RNAi in the absence of the global gene silencing noted upon the introduction of long dsRNAs.

Although protection against HIV-1 after transfection of cells with siRNA duplexes or siRNA expression plasmids has been reported by several groups (5, 15, 18, 23, 33, 44), this strategy has several problems, including the transient nature of the antiviral effect that is produced and the difficulty of introducing nucleic acids into primary cells. In addition, targeting of a virus that displays the sequence variability of HIV-1 by using a highly sequence-specific reagent such as an siRNA might be predicted to rapidly lead to the selection of resistant viral variants. To address these concerns, we have identified an siRNA that can effectively block the expression of the cellular protein CCR5, a key coreceptor for both initial infection and subsequent dissemination of primary HIV-1 isolates in humans (38). We have also developed a lentivirus vector that can express siRNAs, targeted either to CCR5 or the essential HIV-1 *tat* gene, at a level sufficient to confer a stable antiviral phenotype on both cell lines and primary macrophages. These data

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suggest that it may be possible to confer a stable phenotype of virus resistance on cells and tissues in vivo by targeting either cellular genes that serve as essential viral cofactors or highly conserved viral RNA sequences.

MATERIALS AND METHODS

Plasmid construction. The mammalian expression plasmids pSuper, pHIV/Tat, pcTat, pcRev, pBC12/CMV/β-gal, and pHIT/G have been described previously (3, 5, 10, 26). The pHIV/SynTat expression plasmid is identical to pHIV/Tat, except that the wild-type *tat* gene has been replaced with a previously described synthetic *tat* gene predicted to encode the same protein product (1, 46). In addition, the HIV-1 proviral clones pNL-ADA, pNL-Luc-ADA, pNL-Luc-HXB, and pNL-Luc-1549 and plasmids expressing human CD4 (pCMV5/CD4), CXCR4 (pCMV5/CXCR4), and CCR5 (pCMV5/CCR5) have also been described previously (6, 48).

A 1,471-bp fragment of pNL-Luc-ADA encoding *vpu*, the first exon of *tat* and *rev*, and part of *env* and *vpr* was deleted by digestion with *Sal*I and *Nhe*I. The sequence encoding the Vpr carboxy terminus was then restored by ligating a PCR-derived DNA fragment into the *Sal*I and *Nhe*I sites. The PCR primers used also inserted unique *Sac*II and *Xma*I sites immediately downstream of *vpr*. Most of the 3′ long terminal repeat (LTR) U3 region (positions −418 to −18) was then deleted by a recombinant PCR with primers that introduced unique *Cla*I and *Xba*I sites. A cytomegalovirus immediate-early promoter linked to the selectable blasticidin resistance (*blr*) marker was then inserted between unique *Bam*HI and *Xho*I sites to generate pNL-SIN-CMV-BLR. These additional manipulations removed further segments of the viral *env*, *rev*, and *nef* genes, as well as the *luc* indicator gene present in pNL-Luc-ADA. However, the HIV-1 Rev response element was left intact (Fig. 1A).

Oligonucleotides encoding siRNAs directed against residues 84 to 111 in the HIV-1 *tat* open reading frame (ORF) (5′-AAGTGTTGCTTTCATTGCCAAG TTTGTT-3′) or residues 953 to 979 in the human *ccr5* ORF (5′-AACGCTTC TGCAAATGCTGTTCTATTT-3′) were cloned into pSUPER essentially as previously described (3) to generate pH1-siTat and pH1-siCCR5, respectively. DNA fragments containing the H1 promoter (31) and sequences encoding the Tat- and CCR5-specific siRNA precursors were excised from pH1-siTat and pH1-siCCR5 by digestion with *Cla*I and *Xba*I and subcloned into the same sites in pNL-SIN-CMV-BLR to generate pNL-H1-siTat and pNL-H1-siCCR5, respectively. A similar lentivirus vector containing the H1 promoter but lacking any siRNA precursor, termed pNL-H1, was generated in parallel as a negative control.

Primary monocyte culture. Peripheral blood mononuclear cells from healthy, HIV-1-negative donors were isolated by Ficoll-Hypaque gradient centrifugation. The cells were then resuspended in Dulbecco's modified Eagle medium and plated at 2×10^6 per well in 24-well tissue culture plates. After 3 h of culture, adherent cells were washed extensively with phosphate-buffered saline (PBS) and then cultured in Dulbecco modified Eagle medium supplemented with 10% human AB serum (Sigma) and 1,000 U of macrophage colony-stimulating factor (R&D Systems) for 1 week to allow differentiation into monocyte-derived macrophages (MDM).

Preparation of virus stocks and transduced cells. Human 293T cells were maintained as previously described (5). To prepare HIV-1 stocks, 293T cells were transfected with 2 μg of pNL-Luc-ADA, pNL-Luc-HXB, or pNL-Luc-1549 as previously described (48). Stocks of vesicular stomatitis virus glycoprotein G (VSV-G)-pseudotyped HIV-1 virions were produced by transfection of 293T cells with 1.5 μg of pNL-ADA and 0.5 μg of pHIT/G. HIV-1-based lentivirus vectors expressing siRNAs were prepared by cotransfection of 293T cells with 1.8 μg of the lentivirus vector, 0.1 μg of pcRev, 0.1 μg of pcTat, and 50 ng of pHIT/G. The culture medium was replaced 16 h later, and supernatant media were harvested ~40 h posttransfection and passed through a 0.45-μm-pore-size filter. Virus yields were measured by p24 Gag antigen capture enzyme-linked immunosorbent assay (NEN Life Science).

RNA and protein expression analysis. Total RNA was prepared from 293T cells transfected with a pSUPER-derived plasmid (pH1 plasmids), or from 293T cells that had been transduced with a lentivirus vector, by using Trizol in accordance with the manufacturer's (Gibco BRL) instructions. Primer extension reactions were performed with 10 μg of total RNA with the primer extension system-avian myeloblastosis virus reverse transcriptase (Promega) in accordance with the manufacturer's instructions. Extension products were analyzed by electrophoresis through denaturing polyacrylamide gels and visualized by autoradiography.

Northern analysis was performed essentially as previously described (5). Total RNA (30 μg) was separated through a 1% agarose gel containing formaldehyde and transferred to a Hybond-N membrane (Amersham). RNA was fixed by UV cross-linking with a Stratalinker (Stratagene). HIV-1, CCR5, and CD4 mRNAs were detected with ^{32}P-labeled random primed DNA probes.

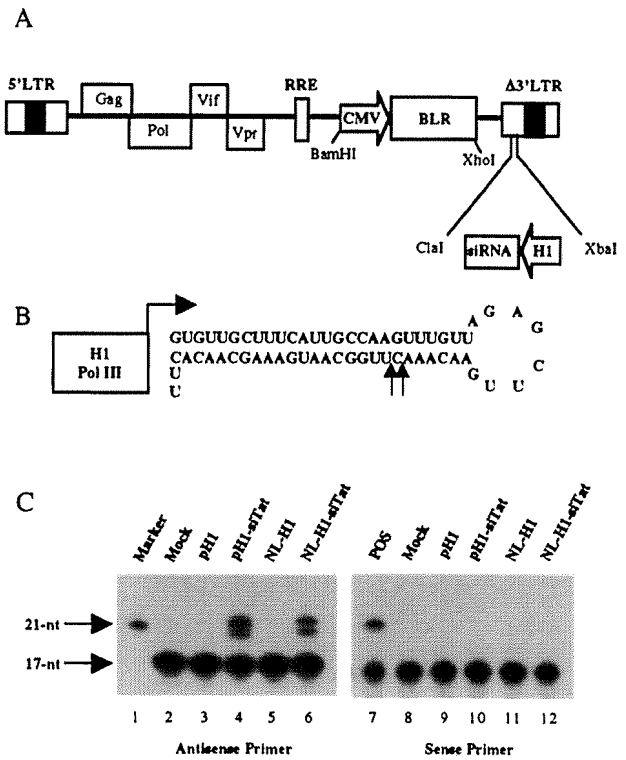

FIG. 1. Expression of siRNAs by a lentivirus vector. (A) Schematic representation of the NL-SIN-CMV-BLR lentivirus vector. The blasticidin resistance (*blr*) gene was introduced as a selectable marker. Unique restriction enzyme sites are indicated. An siRNA expression cassette containing the human H1 promoter can be introduced in place of most of the 3′ LTR U3 region. (B) Predicted stem-loop structure of the Tat siRNA precursor encoded by the pNL-H1-siTat lentivirus vector. Arrows indicate the 5′ ends of the mature siRNAs encoded by this vector. (C) Primer extension analysis. pH1, cells transfected with 1 μg of pSuper; pH1-siTat, cells transfected with 1 μg of pH1-siTat; NL-H1, cells transduced with the NL-H1 lentivirus vector; NL-H1-siTat, cells transduced with NL-H1-siTat. A probe that detects the 3′ or antisense strand was used for lanes 2 to 6, and a probe specific for the 5′ or sense strand was used for lanes 7 to 12. Lane 1 contained a labeled 21-nt RNA marker, while lane 7 contained a synthetic 21-nt RNA identical to the first 21 nt of the 5′ arm of the siRNA precursor as a positive (POS) control. Mock, mock-transfected wild-type 293T cells.

Western analysis of protein expression in transfected 293T cells was performed as previously described (5). Briefly, cell lysates prepared from transfected 293T cells were boiled for 5 min in sodium dodecyl sulfate loading buffer containing 10% 2-mercaptoethanol. Proteins were separated by gel electrophoresis and transferred to a nitrocellulose membrane (Amersham). Western analysis was performed with a rabbit polyclonal antiserum directed against the HIV-1 Tat protein (1:1,000) or a mouse monoclonal antibody directed against β-galactosidase (β-gal; 1:2,000; Promega). The anti-Tat serum used was raised with recombinant full-length Tat and has been provided to the AIDS Research and Reference Reagent Program (catalog no. 705). Bound antibodies were detected with horseradish peroxide-conjugated anti-rabbit or anti-mouse secondary antibodies (Amersham) at a 1:3,000 dilution and visualized by chemiluminescence.

For quantitation of cell surface CCR5, transduced 293T cells were transfected with pCMV5/CCR5 and pEGFP. At 48 h after transfection, cells were stained with a monoclonal anti-CCR5 serum conjugated with Cy-Chrome (BD PharMingen) on ice for 30 min. The cells were then washed extensively with PBS, fixed

with 4% formaldehyde in PBS, and then analyzed by fluorescence-activated cell sorting (FACS) on a FACScan.

Luciferase reporter virus assay. Lentivirus vector-transduced 293T cells were transfected with 100 ng of pCMV5/CD4 and 20 ng of pCMV5/CCR5 or pCMV5/CXCR4 by using calcium phosphate. In some experiments, cells were additionally transfected with 10 ng of pHIV/Tat or pHIV/SynTat or with the parental pBC12/CMV plasmid as a negative control. At 48 h after transfection, the cells were infected with 20 ng of p24 Gag antigen of the NL-Luc-ADA virus for pCMV5/CCR5-transfected cells or the NL-Luc-HXB virus for pCMV5/CXCR4-transfected cells. After 48 h, cells were lysed in 200 μl of lysis buffer (Promega) and luciferase activities were determined with a Lumat LB9501 luminometer.

For infection of macrophages, 7-day-old cultures of MDM were infected overnight with 100 ng of p24 antigen from one of the NL-H1 lentivirus vectors. On the next day, cells were washed with PBS and placed in fresh medium. The cells were allowed to recover for 1 further day and then subjected to a second round of infection with a lentivirus vector. Two days after the second infection, the transduced MDM were infected with 20 ng of p24 antigen from the luciferase reporter virus NL-Luc-ADA or NL-Luc-1549. The cells were harvested 72 h later for luciferase assay as described above.

RESULTS

Construction of a lentivirus siRNA expression vector. Although we and others have shown that transfection of siRNAs, or of siRNA expression plasmids, can effectively protect cells against HIV-1 (5, 15, 18, 23, 33, 44), this protection is only temporary. To address this concern, we constructed a lentivirus vector, pNL-SIN-CMV-BLR, that can be used to stably transduce cultured or primary cells with an siRNA expression cassette (Fig. 1A).

pNL-SIN-CMV-BLR was derived from pNL-Luc-ADA (48) by deletion of part or all of the HIV-1 *vpu*, *tat*, *rev*, *env*, and *nef* genes, as well as almost all of the U3 region in the 3′ LTR. The viral *gag*, *pol*, *vif*, and *vpr* genes, as well as the 5′ LTR and the Rev response element, were, however, left intact, as were all of the *cis*-acting sequences required for efficient genome packaging, reverse transcription, and integration. In place of the deleted sequences, we inserted a cytomegalovirus immediate-early promoter driving the *blr* selectable marker, as well as unique *Cla*I and *Xba*I sites in the 3′ LTR U3 region. These can be used to insert an siRNA expression cassette consisting of the RNA polymerase III-dependent H1 promoter (3, 31) linked to a sequence designed to form a short RNA stem-loop sequence that can be processed by endogenous dicer to yield an siRNA duplex (3, 34) (Fig. 1). This cassette replaces much of the 3′ LTR U3 region and will therefore be duplicated during reverse transcription to replace the promoter and enhancer sequences in the U3 region of the 5′ LTR, thus making this a self-inactivating double-copy lentivirus expression vector.

The initial siRNA target sequence chosen was derived from nucleotides (nt) 84 to 111 of the HIV-1 *tat* ORF and is shown in Fig. 1B. The sequence inserted into the pSuper siRNA expression plasmid (3) is predicted to give rise to a 26-bp dsRNA stem flanked by an 8-nt terminal loop and a 2-nt 3′ overhang derived from the transcription termination sequence for polymerase III. This expression cassette was excised from the resultant pH1-siTat plasmid by cleavage with *Xba*I and *Cla*I and then inserted into the pNL-SIN-CMV-BLR lentivirus vector to give pNL-H1-siTat (Fig. 1A). The lentivirus vector encoded by pNL-H1-siTat was packaged by cotransfection into 293T cells along with plasmids expressing HIV-1 Tat and Rev cDNAs and VSV-G. Packaged virions (50 ng of p24 Gag) were used to infect 10^6 293T cells, and transductants were selected with 10 μg of blasticidin per ml. Generally, >60% of the infected cells were successfully transduced with this protocol.

Primer extension was used to measure the production of siRNAs from either the initial pH1-siTat expression plasmid in transfected 293T cells or the lentivirus NL-H1-siTat vector in pooled, transduced 293T cells (Fig. 1C). The antisense or 3′ arm of the predicted stem-loop precursor (Fig. 1B) was found to give rise to two overlapping siRNAs 21 or 22 nt in length, measured from the base of the stem, and equivalent levels of each siRNA were observed in both transfected and transduced 293T cells (Fig. 1C, lanes 4 and 6). These two products presumably arise from processing by dicer at two adjacent cleavage sites in the predicted RNA hairpin (Fig. 1B). In contrast, the 5′ or sense arm did not give rise to any detectable siRNA product in vivo, although a synthetic 21-nt RNA homologous to the predicted siRNA did give rise to a readily detectable extension product (Fig. 1C, lane 7). Therefore, the RNA stem-loop structure depicted in Fig. 1B behaves like a micro-RNA stem-loop precursor in that only one arm of the precursor appears to have been stably included in the RISC (14, 17).

Stable expression of an siRNA specific for HIV-1 *tat*. Previously, we have shown that transfection of an siRNA duplex specific for the HIV-1 *tat* gene can effectively block HIV-1 replication in culture (5). To test whether expression of a Tat-specific siRNA from a lentivirus vector would exert a similar phenotype, we transfected 293T cells transduced with NL-H1-siTat or with NL-H1, which is not predicted to express any siRNA, with expression vectors encoding HIV-1 Tat and β-gal. As shown in Fig. 2A, we noted a significant drop in Tat expression, compared to that of the β-gal control, in NL-H1-siTat-transduced cells. To test whether the Tat siRNA would have any effect on HIV-1 replication, we next infected control and transduced 293T cells with HIV-1 virions pseudotyped with VSV-G and then quantified virus replication by measuring the level of secreted p24 Gag protein produced by the infected cells. As shown in Fig. 2B, the NL-H1-siTat-transduced cells were largely incapable of giving rise to progeny virions while control NL-H1-transduced cells or nontransduced 293T cells gave rise to high levels of progeny virions, as measured by p24 enzyme-linked immunosorbent assay of the culture supernatants. As shown in the Northern analysis presented in Fig. 2C, the lack of progeny virion production in the NL-H1-siTat-transduced cells coincided with a large drop in the expression of all HIV-1 mRNA species after infection, as would be predicted if Tat function was largely blocked.

To demonstrate that the observed inhibition of HIV-1 replication was indeed specific, we next wished to demonstrate that mutation of the siRNA target site in the HIV-1 *tat* gene would rescue virus replication. Unfortunately, introduction of several point mutations into this sequence in the context of a full-length HIV-1 proviral clone resulted in an intrinsically defective virus, even though these mutations were designed not to affect the sequence of the encoded Tat protein (data not shown).

As an alternative approach to the demonstration of specificity, we therefore investigated whether HIV-1 proviral transcription could be rescued by cotransfection of an expression plasmid encoding a *tat* gene in which the siRNA target sequence was mutated, but not by a similar vector encoding wild-type Tat. The expression plasmid chosen, pHIV/SynTat, is

To confirm this prediction, we first transfected NL-H1-siTat-transduced or control NL-H1-transduced cells with either pHIV/Tat (encoding wild-type Tat) or pHIV/SynTat (carrying the modified *tat* gene), as well as with pBC12/CMV/β-gal as an internal control. As can be seen in Fig. 3B, Tat expression from the pHIV/SynTat vector, unlike Tat expression from pHIV/Tat, was indeed resistant to the siRNA expressed in the NL-H1-siTat-transduced cells.

To determine whether HIV-1 proviral transcription could be selectively rescued by the mutant *tat* gene carried by pHIV/SynTat, we transfected NL-H1-siTat-transduced cells with expression plasmids encoding CD4 and CCR5 and 10 ng of either pHIV/Tat or pHIV/SynTat or a negative control plasmid. NL-H1-transduced cells transfected with vectors encoding CD4 and CCR5 only served as a positive control, while nontransfected NL-H1 cells served as a negative control. At 48 h later, these cultures were infected with the previously described NL-Luc-ADA indicator virus containing the *luc* indicator gene substituted for *nef*. Importantly, the *luc* mRNA encoded by this virus is expressed in the absence of Rev function and is not predicted to contain the *tat* siRNA target sequence. As shown in Fig. 3C, transfection with the pHIV/SynTat plasmid, bearing a mutated siRNA target sequence, indeed fully rescued luciferase expression by the infecting indicator virus, while the wild-type Tat expression plasmid pHIV/Tat had little or no positive effect. We therefore concluded that the observed inhibition of HIV-1 replication and gene expression was indeed due to the specific degradation of viral mRNAs bearing the *tat* siRNA target sequence.

An important goal of this research was to confer a stable, virus-resistant phenotype on cells transduced with the lentivirus siRNA expression vector, rather than the transient resistance characteristic of cells transfected with siRNAs. To test whether resistance to HIV-1 replication was indeed stable, we compared the virus resistance of NL-H1-siTat-transduced cells after 1 week versus 12 weeks in culture. In this experiment, the transduced 293T cells were first transfected with expression plasmids encoding CD4 and either CCR5 or CXCR4. After 48 h, we then infected the transduced, transfected 293T cells with the previously described R5-tropic NL-Luc-ADA indicator virus or the X4-tropic NL-Luc-HXB indicator virus (6, 48). As shown in Fig. 4, 293T cells transduced with NL-H1-siTat remained nonpermissive for productive HIV-1 infection by either indicator virus after 12 weeks in culture, in contrast to the control NL-H1-transduced cells. Primer extension analyses revealed that the level of expression of the siRNA encoded by the NL-H1-siTat provirus was essentially the same after 1 week or 12 weeks in culture (data not shown). We therefore concluded that the antiviral state induced by the lentivirus NL-H1-siTat siRNA expression vector was stable over the period tested.

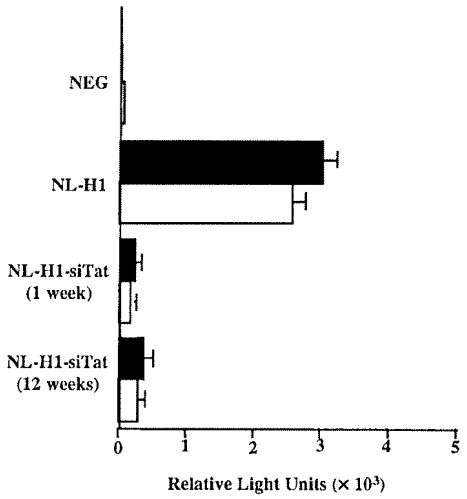

FIG. 4. Stable inhibition of HIV-1 gene expression in NL-H1-siTat-transduced cells. 293T cells transduced with NL-H1-siTat were cultured under selection for either 1 week or 12 weeks. Transduced cells were then transfected with expression plasmids encoding CD4 and CCR5 or CD4 and CXCR4 and infected with the R5-tropic NL-Luc-ADA or X4-tropic NL-Luc-HXB indicator virus, and induced luciferase activities were determined 2 days later. The average of three experiments and the standard deviation are indicated. NEG, negative control.

Inhibition of CCR5 expression with a lentivirus siRNA expression vector. Although the NL-H1-siTat lentivirus vector can potently inhibit HIV-1 replication (Fig. 2 to 4), this represents an idealized system in that the siRNA is designed to target the sequence of a cloned HIV-1 provirus. However, siRNA function can be severely compromised by even a small number of mismatches with the mRNA target sequence (Fig. 3). Given both the known variability of primary HIV-1 gene sequences and the mutability of these sequences in the face of selective pressure, it appears unlikely that siRNAs targeted against HIV-1 would be able to suppress virus replication over the long term in vivo.

To address this issue, we designed siRNAs targeted to the CCR5 coreceptor. CCR5 is critical for HIV-1 transmission between humans and is necessary for infection by the majority of primary HIV-1 isolates (38). Although important for HIV-1 infection, CCR5 is dispensable for human health and well-being, as shown by the existence of individuals who are homozygous for a point mutation that totally blocks CCR5 expression (25).

We identified an siRNA, directed against residues 953 to 979 of the human *ccr5* ORF, that was highly effective at inhibiting CCR5 expression by cotransfection (data not shown). This sequence was incorporated into the stem of a predicted RNA stem-loop structure, expressed under the control of the H1 promoter and then introduced into the pNL-SIN-CMV-BLR lentivirus vector to give pNL-H1-CCR5, as shown in Fig. 1. This lentivirus siRNA expression vector was then packaged in 293T cells and used to infect other 293T cells, and transductants were selected by culture in blasticidin.

To test whether the siRNA expressed from the lentivirus vector would indeed exert an inhibitory effect on CCR5 expression, we transfected pooled 293T cells transduced with NL-H1-siCCR5, with NL-H1 as a control, with plasmids expressing green fluorescent protein (GFP), and with CCR5. At 48 h after transfection, cells were stained with a monoclonal antibody specific for CCR5 and subjected to FACS to quantitate the level of cell surface CCR5 expression on GFP-expressing cells. Although the levels of GFP expression in the different transduced cells were similar (data not shown), the level of CCR5 expression observed was dramatically lower in the cells

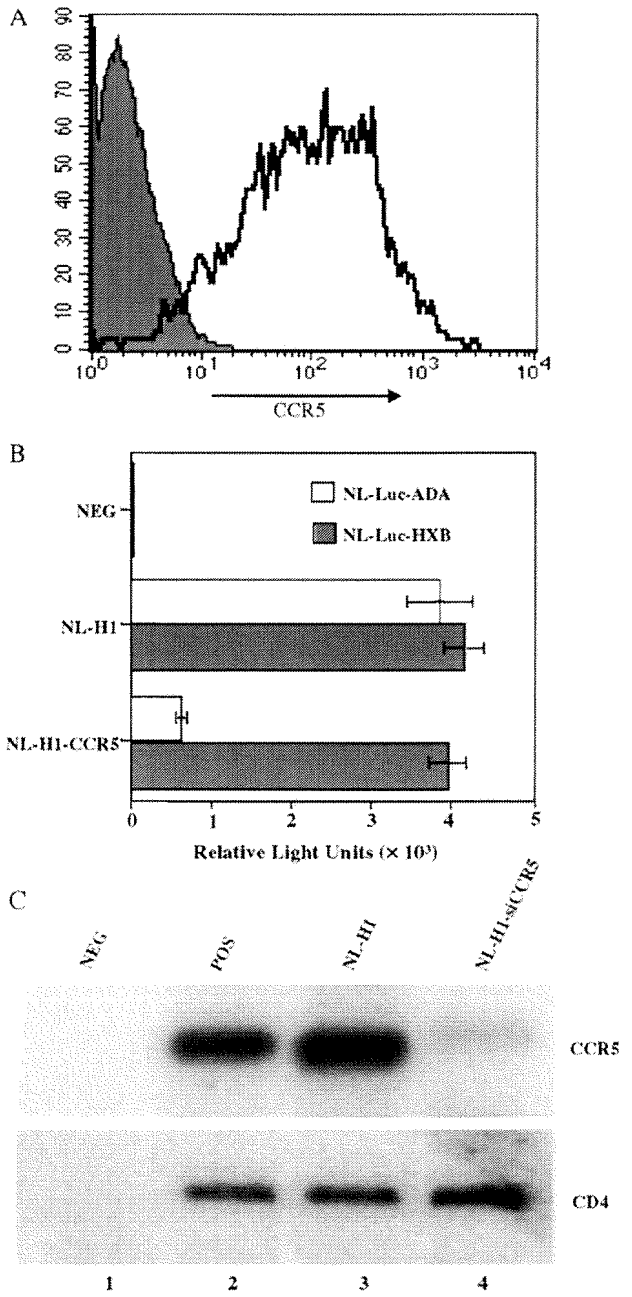

FIG. 5. Inhibition of CCR5 expression by a lentivirus-expressed siRNA. (A) Transduced 293T cells were transfected with plasmids expressing CCR5 and GFP. At 48 h after transfection, the level of cell surface CCR5 expression on GFP-positive cells was analyzed by FACS with an anti-CCR5 monoclonal antibody. CCR5 expression on NL-H1-siCCR5-transduced cells is represented by shading, and that on NL-H1-transduced cells is represented by the open area. (B) Transduced 293T cells were transfected with expression plasmids encoding CD4 and CCR5 or CD4 and CXCR4. At 48 h after transfection, $CD4^+$ $CCR5^-$ cells were infected with NL-Luc-ADA while $CD4^+$ $CXCR4^+$ cells were infected with NL-Luc-HXB. Induced luciferase levels were determined 48 h after infection and are indicated. NEG, nontransfected 293T cells. The average of three experiments and the standard deviation are indicated. (C) Transduced or control 293T cells were transfected with CCR5 and CD4 expression plasmids. At 48 h after transfection, total RNA was extracted and subjected to Northern analysis to measure CCR5 mRNA (top) and CD4 mRNA (bottom) expression. Mock-transfected 293T cells served as a negative control (NEG), while wild-type 293T cells transfected with CCR5 and CD4 served as a positive control (POS).

transduced with NL-H1-siCCR5 than in the control NL-H1-transduced cells (Fig. 5A).

To test whether the siRNA expressed from the NL-H1-siCCR5 lentivirus vector would selectively inhibit infection by R5-tropic HIV-1, we next transfected transduced 293T cells with plasmids expressing CD4 and CCR5 or CD4 and CXCR4. Mock-transfected 293T cells served as a negative control. Two days later, we infected the transfected 293T cells with the R5-tropic indicator virus NL-Luc-ADA or the X4-tropic indicator virus NL-Luc-HXB (48). At 48 h later, the cells were lysed and induced luciferase expression levels were quantitated. As shown in Fig. 3B, NL-Luc-ADA and NL-Luc-HXB were able to infect the control NL-H1-transduced cells with comparable efficiencies, as shown by the similar levels of expression of the virus-encoded luciferase. In contrast, while the NL-H1-siCCR5-transduced cells remained fully permissive for infection by the X4-tropic NL-Luc-HXB indicator virus, infection by the R5-tropic NL-Luc-ADA indicator virus was strongly reduced (Fig. 5B).

If the siRNA expressed in the NL-H1-siCCR5-transduced cells is indeed functional, then it should induce the selective degradation of CCR5 mRNA (14, 17, 30). To test this hypothesis, we prepared total RNA from cultures transfected as described above and performed Northern analysis for either the CCR5 or the CD4 mRNA expressed from the cotransfected plasmids. As shown in Fig. 5C, we observed a marked and specific inhibition of the level of CCR5 mRNA expression in the NL-H1-siCCR5-transduced cells compared to that in control NL-H1-transduced cells, while the level of CD4 mRNA expression remained constant. Together, these data indicate that the CCR5-specific siRNA produced by the NL-H1-siCCR5 lentivirus vector is able to specifically reduce not only CCR5 mRNA and protein expression but also infection by R5-tropic HIV-1.

Inhibition of HIV-1 replication in primary macrophages by siRNAs expressed from lentivirus vectors. The data presented thus far were all obtained with the human cell line 293T, and we were therefore anxious to confirm that the lentivirus siRNA expression vectors would also function in primary cells. We therefore used the lentivirus vectors to transduce primary MDM. It has previously been shown by several groups that these nondividing cells can be effectively infected by HIV-1 or by HIV-1-derived lentivirus vectors (4, 24, 32, 49).

To obtain good levels of infection without selection and without having to concentrate the lentivirus vector preparations and to see if we could observe functional synergy between two distinct lentivirus siRNA expression vectors, we transduced each MDM culture twice over a 2-day interval (Fig. 6). Two days after the second transduction, the MDM were infected with aliquots of the R5-tropic NL-Luc-ADA or the X4-tropic NL-Luc-1549 indicator virus. The *env* gene used in NL-Luc-1549 is derived from a primary X4-tropic HIV-1 strain that, unlike laboratory X4-tropic HIV-1 isolates, is able to

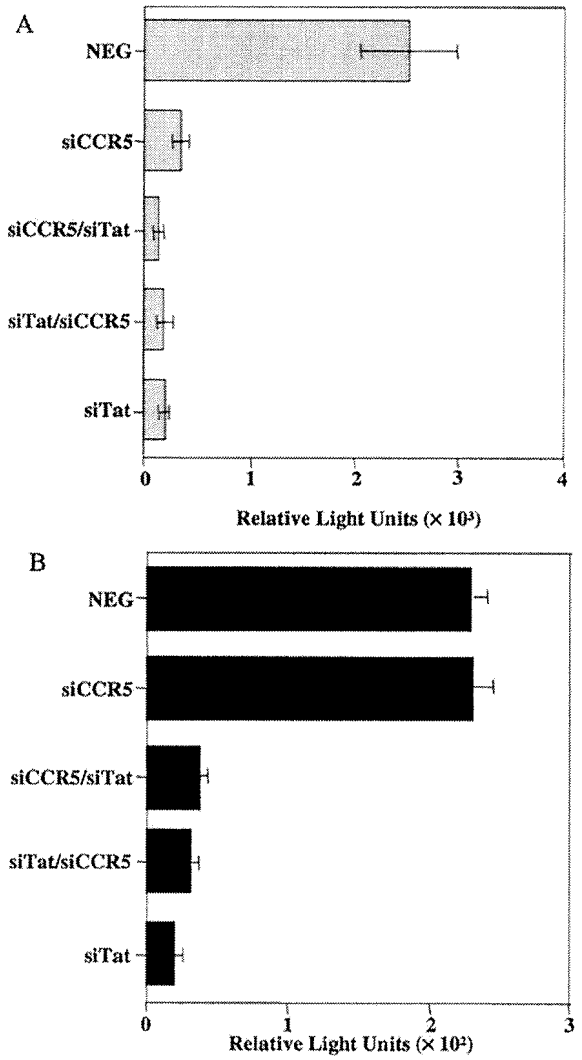

FIG. 6. Single-cycle assay of HIV-1 replication in primary macrophages. MDM were cultured for 7 days in the presence of macrophage colony-stimulating factor and then transduced twice with a lentivirus vector stock over a 2-day period. The order of vector infection is shown; e.g., siCCR5 denotes MDM transduced twice with NL-H1-siCCR5, while siCCR5/siTat denotes MDM transduced first with NL-H1-siCCR5 and then with NL-H1-siTat. Two days later, the transduced MDM were infected with the R5-tropic reporter virus NL-Luc-ADA (A) or X4-tropic reporter virus NL-Luc-1549 (B) and induced luciferase levels were measured a further 48 h later. Doubly NL-H1-transduced MDM served as the negative control (NEG). The average of three experiments and the standard deviation are indicated.

infect primary macrophages with exclusively endogenous CXCR4 as a coreceptor (48).

As shown in Fig. 6A, we observed efficient inhibition of MDM infection by the R5-tropic NL-Luc-ADA indicator virus after transduction with NL-H1-siTat, NL-H1-siCCR5, or both lentivirus vectors. The efficiency of inhibition by NL-H1-siTat was such that it was not possible to see any synergy with NL-H1-siCCR5 under these assay conditions. In contrast to the R5-tropic NL-Luc-ADA indicator virus, infection of primary macrophages by the X4-tropic NL-Luc-1549 virus was entirely unaffected by transduction of the cells with NL-H1-siCCR5, as predicted. However, this virus remained fully susceptible to inhibition by NL-H1-siTat (Fig. 6B). We therefore concluded that these lentivirus siRNA expression vectors are capable of expressing siRNAs in primary macrophages at levels sufficient to effectively block functional expression of the cellular *ccr5* or viral *tat* gene product.

DISCUSSION

While there has been considerable excitement over the potential of RNAi as a treatment for virus-induced diseases, this potential will only be achievable if the relevant nucleic acids can be effectively delivered to the appropriate cells in vivo, if an effective level of expression of the siRNAs can be maintained over a long period, and if the target virus is not able to rapidly undergo selection for variants that are resistant to the siRNAs used.

While we and others have previously reported excellent protection against HIV-1 and other viruses in cell lines and primary cells in culture (2, 5, 11, 12, 15, 18, 19, 23, 33, 36, 41, 44, 50), the approach used, i.e., primarily transfection of siRNA duplexes, gives rise to only transient inhibition. To address this concern, we have developed a simple lentivirus vector that can effectively and stably express siRNAs at a level sufficient to block HIV-1 replication in culture (Fig. 1). Although the pNL-SIN-CMV-BLR vector may not be sufficiently defective to be suitable for use in humans, these data nevertheless provide proof of principle for the concept that stable expression of siRNAs, and hence a stable antiviral phenotype, can be achieved with lentivirus vectors or other viral vectors.

A second concern noted above is the issue of viral variability and the resultant ability of viruses to escape inhibition by siRNAs targeted to even highly conserved regions in the viral genome. While it might, in principle, be possible to design siRNAs targeted to multiple critical viral sequences that, in combination, would provide long-term protection, a possible alternative strategy is to target a cellular gene product that is essential for virus replication but dispensable to the host. The human *ccr5* gene provides an ideal example of such a target, as it is critical for infection by most primary HIV-1 isolates yet apparently of little or no importance to the human host (25, 38).

With the lentivirus vector siRNA expression strategy, we were indeed able to effectively and specifically reduce CCR5 protein and mRNA expression in transduced cells (Fig. 5). More importantly, this approach allowed us to selectively inhibit infection by R5-tropic, but not X4-tropic, HIV-1 isolates of not only cell lines but also primary macrophages (Fig. 5B and 6). These data therefore confirm and extend the recent report by Qin et al. (35) showing that a lentivirus-expressed siRNA targeted to CCR5 can inhibit infection of primary T cells by R5-tropic HIV-1. Together, these reports suggest that this strategy has the potential to block HIV-1 infection in vivo in a manner that cannot be overcome simply by a 1- or 2-nt change in the viral siRNA target sequence (12). Of course, HIV-1 might still be able to escape this inhibitory effect by switching to another coreceptor, such as CXCR4 (25, 38). While this certainly represents a serious concern, we note that it may also be possible to simultaneously knock down CXCR4

expression in adult patients with RNAi without serious side effects for the host and to thereby preclude this avenue of viral escape (29).

Although the focus of this report has been the potential of lentivirus siRNA expression vectors for rendering cells nonpermissive for HIV-1 replication, these vectors also have the potential to induce the stable knockdown of important cellular genes unrelated to the HIV-1 life cycle in either cultured cells or experimental animals (39, 42, 47). We therefore intend to make the pNL-SIN-CMV-BLR lentivirus vector, as well as a variant expressing *gfp* in place of *blr*, available to academic researchers on request.

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REFERENCES

1. **Adams, S. E., I. D. Johnson, M. Braddock, A. J. Kingsman, S. M. Kingsman, and M. Edwards.** 1988. Synthesis of a gene for the HIV transactivator protein TAT by a novel single stranded approach involving *in vivo* gap repair. Nucleic Acids Res. **16:**4287–4298.
2. **Bitko, V., and S. Barik.** 2001. Phenotypic silencing of cytoplasmic genes using sequence-specific double-stranded short interfering RNA and its application in the reverse genetics of wild type negative-strand RNA viruses. BMC Microbiol. **1:**34.
3. **Brummelkamp, T. R., R. Bernards, and R. Agami.** 2002. A system for stable expression of short interfering RNAs in mammalian cells. Science **296:**550–553.
4. **Bukrinsky, M. I., S. Haggerty, M. P. Dempsey, N. Sharova, A. Adzhubei, L. Spitz, P. Lewis, D. Goldfarb, M. Emerman, and M. Stevenson.** 1993. A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. Nature **365:**666–669.
5. **Coburn, G. A., and B. R. Cullen.** 2002. Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. J. Virol. **76:**9225–9231.
6. **Connor, R. I., B. K. Chen, S. Choe, and N. R. Landau.** 1995. VPR is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. Virology **206:**935–944.
7. **Covey, S. N., N. S. Al-Kaff, A. Langara, and D. S. Turner.** 1997. Plants combat infection by gene silencing. Nature **385:**781–782.
8. **Elbashir, S. M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl.** 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature **411:**494–498.
9. **Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello.** 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature **391:**806–811.
10. **Fouchier, R. A. M., B. E. Meyer, J. H. M. Simon, U. Fischer, and M. H. Malim.** 1997. HIV-1 infection of non-dividing cells: evidence that the amino-terminal basic region of the viral matrix protein is important for Gag processing but not for post-entry nuclear import. EMBO J. **16:**4531–4539.
11. **Ge, Q., M. T. McManus, T. Nguyen, C.-H. Shen, P. A. Sharp, H. N. Eisen, and J. Chen.** 2003. RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. Proc. Natl. Acad. Sci. USA **100:**2718–2723.
12. **Gitlin, L., S. Karelsky, and R. Andino.** 2002. Short interfering RNA confers intracellular antiviral immunity in human cells. Nature **418:**430–434.
13. **Hammond, S. M., E. Bernstein, D. Beach, and G. J. Hannon.** 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature **404:**293–295.
14. **Hannon, G. J.** 2002. RNA interference. Nature **418:**244–251.
15. **Hu, W.-Y., C. P. Myers, J. M. Kilzer, S. L. Pfaff, and F. D. Bushman.** 2002. Inhibition of retroviral pathogenesis by RNA interference. Curr. Biol. **12:** 1301–1311.
16. **Hutvágner, G., J. McLachlan, A. E. Pasquinelli, É. Bálint, T. Tuschl, and P. D. Zamore.** 2001. A cellular function for the RNA-interference enzyme dicer in the maturation of the *let-7* small temporal RNA. Science **293:**834–838.
17. **Hutvágner, G., and P. D. Zamore.** 2002. RNAi: nature abhors a double-strand. Curr. Opin. Genet. Dev. **12:**225–232.
18. **Jacque, J.-M., K. Triques, and M. Stevenson.** 2002. Modulation of HIV-1 replication by RNA interference. Nature **418:**435–438.
19. **Kapadia, S. B., A. Brideau-Andersen, and F. V. Chisari.** 2003. Interference of hepatitis C virus RNA replication by short interfering RNAs. Proc. Natl. Acad. Sci. USA **100:**2014–2018.
20. **Ketting, R. F., S. E. Fischer, E. Bernstein, T. Sijen, G. J. Hannon, and R. H. Plasterk.** 2001. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev. **15:**2654–2659.
21. **Ketting, R. F., T. H. Haverkamp, H. G. van Luenen, and R. H. Plasterk.** 1999. Mut-7 of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNase D. Cell **99:**133–141.
22. **Knight, S. W., and B. L. Bass.** 2001. A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in C. elegans. Science **293:**2269–2271.
23. **Lee, N. S., T. Dohjima, G. Bauer, H. Li, M.-J. Li, A. Ehsani, P. Salvaterra, and J. Rossi.** 2002. Expression of small interfering RNAs targeted against HIV-1 *rev* transcripts in human cells. Nat. Biotechnol. **19:**500–504.
24. **Lewis, P., M. Hensel, and M. Emerman.** 1992. Human immunodeficiency virus infection of cells arrested in the cell cycle. EMBO J. **8:**3053–3058.
25. **Liu, R., W. A. Paxton, S. Choe, D. Ceradini, S. R. Martin, R. Horuk, M. E. MacDonald, H. Stuhlmann, R. A. Koup, and N. R. Landau.** 1996. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell **86:**367–377.
26. **Malim, M. H., J. Hauber, R. Fenrick, and B. R. Cullen.** 1988. Immunodeficiency virus *rev trans*-activator modulates the expression of the viral regulatory genes. Nature **335:**181–183.
27. **Manche, L., S. R. Green, C. Schmedt, and M. B. Mathews.** 1992. Interactions between double-stranded RNA regulators and the protein kinase DAI. Mol. Cell. Biol. **12:**5238–5248.
28. **Martinez, J., A. Patkaniowska, H. Urlaub, R. Lührmann, and T. Tuschl.** 2002. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell **110:**563–574.
29. **Martinez, M. A., A. Gutierrez, M. Armand-Ugon, J. Blanco, M. Parera, J. Gomez, B. Clotet, and J. A. Este.** 2002. Suppression of chemokine receptor expression by RNA interference allows for inhibition of HIV-1 replication. AIDS **16:**2385–2390.
30. **McManus, M. T., and P. A. Sharp.** 2002. Gene silencing in mammals by short interfering RNAs. Nat. Rev. **3:**737–747.
31. **Myslinski, E., J.-C. Amé, A. Krol, and P. Carbon.** 2001. An unusually compact external promoter for RNA polymerase III transcription of the human H1RNA gene. Nucleic Acids Res. **29:**2502–2509.
32. **Naldini, L., and I. M. Verma.** 2000. Lentiviral vectors. Adv. Virus Res. **55:**599–609.
33. **Novina, C. D., M. F. Murray, D. M. Dykxhoorn, P. J. Beresford, J. Riess, S.-K. Lee, R. G. Collman, J. Lieberman, P. Shankar, and P. A. Sharp.** 2002. siRNA-directed inhibition of HIV-1 infection. Nat. Med. **8:**681–686.
34. **Paddison, P. J., A. A. Caudy, E. Bernstein, G. J. Hannon, and D. S. Conklin.** 2002. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev. **16:**948–958.
35. **Qin, X.-F., D. S. An, I. S. Y. Chen, and D. Baltimore.** 2003. Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. Proc. Natl. Acad. Sci. USA **100:**183–188.
36. **Randall, G., A. Grakoui, and C. M. Rice.** 2003. Clearance of replicating hepatitis C virus replicon RNAs in cell culture by small interfering RNAs. Proc. Natl. Acad. Sci. USA **100:**235–240.
37. **Ratcliff, F., B. D. Harrison, and D. C. Baulcombe.** 1997. A similarity between viral defense and gene silencing in plants. Science **276:**1558–1560.
38. **Ross, T. M., P. D. Bieniasz, and B. R. Cullen.** 1999. Role of chemokine receptors in HIV-1 infection and pathogenesis. Adv. Virus Res. **52:**233–267.
39. **Rubinson, D. A., C. P. Dillon, A. V. Kwiatkowski, C. Sievers, L. Yang, J. Kopinja, M. Zhang, M. T. McManus, F. B. Gertler, M. L. Scott, and L. Van Parijs.** 2003. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat. Genet. **33:**401–406.
40. **Schwarz, D. S., G. Hutvágner, B. Haley, and P. D. Zamore.** 2002. Evidence that siRNAs function as guides, not primers, in the *Drosophila* and human RNAi pathways. Mol. Cell **10:**537–548.
41. **Shlomai, A., and Y. Shaul.** 2003. Inhibition of hepatitis B virus expression and replication by RNA interference. Hepatology **37:**764–770.
42. **Stewart, S. A., D. M. Dykxhoorn, D. Palliser, H. Mizuno, E. Y. Yu, D. S. An, D. M. Sabatini, I. S. Y. Chen, W. C. Hahn, P. A. Sharp, R. A. Weinberg, and C. D. Novina.** 2003. Lentivirus-delivered stable gene silencing by RNAi in primary cells. RNA **9:**493–501.
43. **Sui, G., C. Soohoo, E. B. Affar, F. Gay, Y. Shi, W. C. Forrester, and Y. Shi.** 2002. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc. Natl. Acad. Sci. USA **99:**5515–5520.
44. **Surabhi, R. M., and R. B. Gaynor.** 2002. RNA interference directed against viral and cellular targets inhibits human immunodeficiency virus type 1 replication. J. Virol. **76:**12963–12973.
45. **Tabara, H., M. Sarkissian, W. G. Kelly, J. Fleenor, A. Grishok, L. Timmons, A. Fire, and C. C. Mello.** 1999. The *rde-1* gene, RNA interference and transposon silencing in *C. elegans*. Cell **99:**123–132.

46. **Tiley, L. S., P. H. Brown, and B. R. Cullen.** 1990. Does the human immunodeficiency virus Tat *trans*-activator contain a discrete activation domain? Virology **178:**560–567.
47. **Tiscornia, G., O. Singer, M. Ikawa, and I. M. Verma.** 2003. A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. Proc. Natl. Acad. Sci. USA **100:**1844–1848.
48. **Tokunaga, K., M. I. Greenberg, M. A. Morse, R. I. Cumming, H. K. Lyerly, and B. R. Cullen.** 2001. Molecular basis for cell tropism of CXCR4-dependent human immunodeficiency virus type 1 isolates. J. Virol. **75:**6776–6785.
49. **Weinberg, J. B., T. J. Matthews, B. R. Cullen, and M. H. Malim.** 1991. Productive human immunodeficiency virus type 1 (HIV-1) infection of nonproliferating human monocytes. J. Exp. Med. **174:**1477–1482.
50. **Wilson, J. A., S. Jayasena, A. Khvorova, S. Sabatinos, I. G., Rodrigue-Gervais, S. Arya, F. Sarangi, M. Harris-Brandts, S. Beaulieu, and C. D. Richardson.** 2003. RNA interference blocks gene expression and RNA synthesis from hepatitis C replicons propagated in human liver cells. Proc. Natl. Acad. Sci. USA **100:**2783–2788.
51. **Zeng, Y., E. J. Wagner, and B. R. Cullen.** 2002. Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. Mol. Cell **9:**1327–1333.

RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription

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Influenza A virus causes widespread infection in the human respiratory tract, but existing vaccines and drug therapy are of limited value. Here we show that short interfering RNAs (siRNAs) specific for conserved regions of the viral genome can potently inhibit influenza virus production in both cell lines and embryonated chicken eggs. The inhibition depends on the presence of a functional antisense strand in the siRNA duplex, suggesting that viral mRNA is the target of RNA interference. However, siRNA specific for nucleocapsid (NP) or a component of the RNA transcriptase (PA) abolished the accumulation of not only the corresponding mRNA but also virion RNA and its complementary RNA. These siRNAs also broadly inhibited the accumulation of other viral, but not cellular, RNAs. The findings reveal that newly synthesized NP and PA proteins are required for influenza virus transcription and replication and provide a basis for the development of siRNAs as prophylaxis and therapy for influenza infection in humans.

Influenza A virus, a member of the *Orthomyxoviridae* family, causes the most prevalent infection of the respiratory tract in humans (1). In a typical year, 10–20% of the population in the United States is afflicted by the virus, resulting in up to 40,000 deaths (2). In what was one of the most devastating human catastrophes in history, at least 20 million people died worldwide during the 1918 influenza virus pandemic (3). The virulence of influenza A virus results from (*i*) its easy spread by aerosol; (*ii*) its ability to escape from protective immunity by frequent changes in viral antigens (antigenic drift) (4, 5); and (*iii*) the periodic emergence of new virulent strains by reassortment or mixing of RNA segments between viruses from two different species (antigenic shift) (6).

The threat of a new influenza pandemic persists because, despite intensive efforts, existing vaccines and therapy for influenza infection have only a limited value (7). Current vaccines, consisting of either killed virus or recombinant surface glycoproteins, induce only a weak IgG response and, as a result, protection can wane in as little as 6 months (8). In the most susceptible population, elderly and immunocompromised individuals, the efficacy of vaccination is merely 39% (2, 8). In addition, the existing vaccines have to be reformulated almost every year because the viral antigens [hemagglutinin (HA) and neuraminidase (NA)] that elicit protective antibodies usually undergo changes, rendering the previous year's vaccine ineffective against any new virus subtype. Similarly, although four antiviral drugs have been approved in the United States for treatment and/or prophylaxis of influenza, their use is limited because of severe side effects and the possible emergence of resistant viruses (9).

RNA interference (RNAi) is a process by which double-stranded RNA (dsRNA) directs sequence-specific degradation of messenger RNA (mRNA) (10–12). This phenomenon was initially observed in *Caenorhabditis elegans* (13), in plants (10, 14), and, recently, in mammalian cells (15). In plants, it is an evolutionarily conserved response to virus infection. Naturally occurring RNAi is initiated by the dsRNA-specific endonuclease Dicer-RDE-1, which processively cleaves long dsRNA into double-stranded fragments between 21 and 25 nt in length, termed short interfering RNA (siRNA) (15). siRNAs are then incorporated into a protein complex that recognizes and cleaves target mRNAs. Studies have shown that in mammalian cells, RNAi can be triggered by introducing synthetic 21-nt siRNA duplexes into the cells (16), bypassing the requirement for Dicer-RDE-1-mediated processing of long dsRNA. Because 21-nt siRNAs are too short to induce an IFN response in mammalian cells (16, 17), yet still able to confer transient interference of gene expression in a sequence-specific manner, they represent a previously unrecognized class of molecules that may have significant medical applications.

We report here the identification of siRNAs that can potently inhibit influenza virus production in both cell lines and embryonated chicken eggs. We show that inhibition by the most potent siRNAs is a result of both sequence-specific interference with viral mRNA accumulation and broad inhibition of all viral RNA transcription.

Materials and Methods

siRNAs. All RNA oligonucleotides were synthesized by Dharmacon Research (Lafayette, CO). The oligonucleotides were deprotected according to the manufacturer's instructions. Equimolar amounts of complementary oligonucleotides were mixed and annealed by heating to 95°C for 5 min, then reducing the temperature slowly by 1°C every 30 sec until 35°C, then by 1°C every min until the temperature reached 5°C. The modified RNA oligonucleotides, in which the 2′-hydroxyl group was replaced with a 2′-O-methyl group in every nucleotide residue, was synthesized by Dharmacon, deprotected, and annealed to complementary strands as above. The resulting siRNA duplexes were analyzed for completion of duplex formation by gel electrophoresis. Sequences of all siRNAs tested are shown in Table 2, which is published as supporting information on the PNAS web site, www.pnas.org.

Viruses and Assays. Influenza viruses A/PR/8/34 (PR8) and A/WSN/33 (WSN), subtypes H1N1, were kindly provided by Peter Palese (Mount Sinai School of Medicine, New York). The viruses were grown in the allantoic cavity of 10-day-old embryonated chicken eggs (Charles River Laboratories, Wilmington, MA) at 37°C. Allantoic fluid was harvested 48 h after virus inoculation and stored at −80°C. Virus titer was measured by hemagglutination or plaque assays. The hemagglutination assay was carried out in V-bottom 96-well plates. Serial 2-fold dilutions of virus samples were mixed with an equal volume of a 0.5%

Abbreviations: vRNA, virion RNA; cRNA, complementary RNA; dsRNA, double-stranded RNA; siRNA, short interfering RNA; RNAi, RNA interference; HA, hemagglutinin; NA, neuraminidase; NP, nucleocapsid protein; MDCK cell, Madin–Darby canine kidney cell; PR8, A/Puerto Rico/8/34 (H1N1); WSN, A/WSN/33 (H1N1); pfu, plaque-forming units; moi, multiplicity of infection; RT, reverse transcription.

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suspension (vol/vol) of chicken erythrocytes (Charles River Laboratories) and incubated on ice for 1 h. Wells containing an adherent, homogeneous layer of erythrocytes were scored as positive. For plaque assays, serial 10-fold dilutions of virus samples were added onto a monolayer of Madin–Darby canine kidney (MDCK) cells in 1% semisolid agar. Two days after infection, plaques were visualized by staining with crystal violet.

Cell Culture and Virus Infection. MDCK cells were grown in DMEM containing 10% heat-inactivated FCS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C under a 5% CO_2/95% air atmosphere. For siRNA introduction, logarithmic-phase MDCK cells were trypsinized, washed, and resuspended in serum-free RPMI medium 1640 at 2×10^7 cells per ml. Cells (0.5 ml) were mixed with siRNA and electroporated at 400 V and 975 μF by using a Gene Pulser apparatus (Bio-Rad). Electroporated cells were divided into three wells of a six-well plate and cultured in DMEM for 8 h. The culture medium was then removed and 100 μl of PR8 or WSN virus in infection medium, consisting of DMEM, 0.3% BSA (Sigma), 10 mM Hepes, 100 units/ml penicillin, and 100 μg/ml streptomycin, was added to each well. After incubation for 1 h at room temperature, 2 ml of infection medium containing 4 μg/ml trypsin was added to each well and the cells were cultured at 37°C under 5% CO_2. At different times after infection, supernatants were harvested from infected cultures and the virus titer was determined.

Virus and siRNA Inoculation in Chicken Embryos. For each inoculation, 30 μl of Oligofectamine (Invitrogen) was diluted with 30 μl of Opti-MEM I (GIBCO). siRNA [2.5 nmol (10 μl)] was mixed with 30 μl of Opti-MEM I and added to diluted Oligofectamine, and the mixture was incubated at room temperature for 30 min. The mixture was then combined with 100 μl of PR8 virus [5,000 plaque-forming units (pfu)/ml] and immediately injected into the allantoic cavity of a 10-day-embryonated chicken egg. The eggs were incubated at 37°C for 17 h and allantoic fluid was harvested to measure virus titer.

RNA Extraction, Reverse Transcription (RT), and Real-Time PCR. MDCK cells (1×10^7) were electroporated with or without siRNA, and were infected 8 h later with PR8 virus. One, 2, and 3 h after infection, culture medium was removed and the cells were lysed by using Trizol reagent (GIBCO). RNA was isolated by following the manufacturer's protocol. RT was carried out by using an Omniscript reverse transcriptase kit (Qiagen, Valencia, CA) in a 20-μl reaction mixture, containing 200 ng of total RNA and specific primers, at 37°C for 1 h. One microliter of RT reaction mixture was then used for real-time PCR by using gene-specific primers, SYBR green PCR Master Mix (Applied Biosystems), and SYBR green I dsDNA binding dye. Before the PCR, the mixture was incubated at 50°C for 2 min and 95°C for 10 min. The reaction was then performed at 95°C for 15 sec and 60°C for 1 min for 50 cycles. All reactions were done in duplicate. The levels of PCR products were monitored with an ABI PRISM 7000 sequence detection system and analyzed with ABI PRISM 7000SDS software (Applied Biosystems). Cycle times were analyzed at a reading of 0.2 fluorescence unit. Cycle times that varied by >1.0 unit between duplicates were discarded. The duplicate cycle times were averaged and normalized to the cycle time of γ-actin. Sequences of RT and PCR primers are shown in Table 3, which is published as supporting information on the PNAS web site.

Results

Design of siRNAs Specific for Influenza A Virus. Influenza A virus has a segmented RNA genome. Three of the eight RNA segments encode three components of the RNA transcriptase (PA, PB1, and PB2) (1). Three additional RNA segments encode the major glycoproteins: HA and NA, and nucleocapsid protein (NP). Each of the remaining two RNA segments encodes two proteins, either M1 or M2, and NS1 or NS2, which function either as viral structural proteins or in the viral life cycle. Among influenza A viruses, 15 HA subtypes and 9 NA subtypes are known. Extensive differences in nucleotide sequences are also present in other genes among virus isolates from humans and different species. To design siRNAs that remain effective despite antigenic drifts and shifts, we focused on regions of the viral genome that are conserved among different subtypes and strains of virus from human, chicken, duck, horse, and swine (the influenza sequence database, www.flu.lanl.gov). Besides having no more than 1 mismatch in the 21 nucleotides among different virus subtypes and strains, the siRNAs designed did not share identity with any known human gene. We designed and tested a total of 20 siRNAs specific for NP, PA, PB1, PB2, M, and NS genes. No siRNA for HA and NA was designed because they contain no stretch of conserved 21 nucleotides, a result of extensive variations in these genes among different virus isolates from humans and various other species.

Inhibition of Influenza Virus Production in a Cell Line. To test whether the synthetic siRNAs inhibited influenza virus production, we first examined their effect in MDCK cells. siRNAs (2.5 nmol) were introduced into MDCK cells (1×10^7) by electroporation, and 8 h later the cells were infected with either PR8 or WSN virus at a multiplicity of infection (moi) of 0.001, 0.01, or 0.1. At different times after infection, culture supernatants were harvested, serially diluted, and assayed to determine the virus titer by using an HA assay. As a control, siRNA specific for GFP (termed GFP-949) was similarly introduced into GFP-expressing MDCK cells, followed by virus infection. Virus titer was assayed as above and GFP expression was assayed by flow cytometry.

As shown in Fig. 1*A*, in mock transfection (no siRNA), virus titers in the infected cultures increased over time, reaching peak values between 48–60 h. Transfection of GFP-949 did not affect virus production at any time point but significantly inhibited GFP expression (data not shown), indicating that siRNA does not interfere nonspecifically with influenza virus production. Transfection of siRNAs specific for influenza virus generated three types of results (Fig. 1 *A* and *D*, and Table 1). First, ≈45% of the siRNAs had no discernable effect on the virus titer, indicating that they were not effective in interfering with influenza virus production in MDCK cells. Second, ≈40% of the siRNAs significantly inhibited virus production. The extent of inhibition varied somewhat, depending on whether PR8 or WSN virus was used. Third, ≈15% of the siRNAs potently inhibited virus production, regardless of whether PR8 or WSN virus was used. When either NP-1496 or PA-2087 siRNA was used, inhibition was so pronounced that culture supernatants lacked detectable HA activity (Fig. 1*A*).

To estimate virus titers more precisely, we performed plaque assays with cultures that were infected with PR8 virus at a moi of 0.001 or 0.1 in the presence or absence of siRNA specific for NP (NP-1496). At a moi of 0.001, $\approx 6 \times 10^5$ pfu/ml was detected in the mock culture, whereas no plaques were detected in the undiluted NP-1496 culture supernatant. As the detection limit of the plaque assay is ≈20 pfu/ml, the inhibition of virus production by NP-1496 is at least 30,000-fold. Even at a moi of 0.1, NP-1496 inhibited virus production 200-fold.

To determine the potency of siRNA, a graded amount of NP-1496 was electroporated into MDCK cells, followed by PR8 infection. Virus titers in the culture supernatants were measured by HA assay. As the amount of siRNA decreased, virus titer increased in the culture supernatants (Fig. 1*B*). However, even when 25 pmol of siRNA was used for electroporation, an ≈4-fold decrease in virus production was detected as compared with

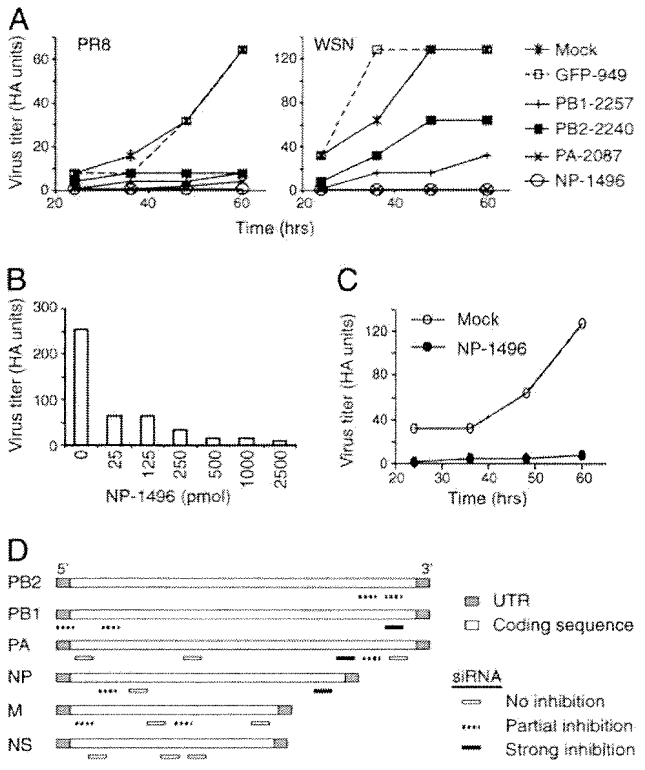

Fig. 1. siRNAs interfere with influenza A virus production in MDCK cells. (*A*) Inhibition of influenza virus production in MDCK cells by selected siRNAs. MDCK cells were first electroporated with siRNAs and then infected 8 h later with PR8 or WSN virus at a moi of 0.01. Viral titers in the culture supernatants at different times after infection were measured by HA assay. HA units are arithmetic means based on titer endpoints of arithmetic dilutions. Virus titers (HA units) from five siRNA-treated cultures are shown over time for both PR8 (*Left*) and WSN (*Right*) infections. NP-1496, etc., are siRNAs specific for different viral genes. For example, NP-1496 indicates an siRNA specific for NP, with the beginning nucleotide at position 1496 of the NP sequence. (*B*) Inhibition of influenza virus production by different doses of siRNA. MDCK cells were transfected with the indicated amount of NP-1496 followed by infection 8 h later with PR8 virus at a moi of 0.1. Virus titer was measured 48 h after infection. Data shown are from one of two experiments. (*C*) Inhibition of influenza virus production by siRNA administered after virus infection. MDCK cells were infected with PR8 virus at a moi of 0.01 and were electroporated 2 h later with NP-1496 (2.5 nmol). Virus titer was measured at the indicated time after infection. Data shown are from one of two experiments. (*D*) Schematic diagrams showing the location of each siRNA in the viral genome and its relative potency in inhibiting influenza virus production in MDCK cells (based on data in Table 1). UTR, untranslated regions.

mock transfection, indicating a high potency of NP-1496 siRNA in inhibiting influenza virus production.

For therapy, siRNA must be able to effectively inhibit an ongoing virus infection. After influenza virus infection, new virions are released in ≈4 h. To eliminate reinfection by newly released virus, MDCK cells were infected with PR8 virus for 2 h and then transfected with NP-1496. With time, virus titer increased steadily after mock transfection, whereas virus titer was only slightly increased in NP-1496-transfected cells (Fig. 1*C*). Thus, administration of siRNA after virus infection can also be effective.

Together, these results show that (*i*) some siRNAs can potently inhibit influenza virus production in MDCK cells (Fig. 1*D*); (*ii*) influenza virus production can be inhibited by siRNAs specific for different viral genes, including those encoding NP, PA, and PB1; and (*iii*) siRNA inhibition can still be initiated in cells with ongoing infection.

Table 1. Effects of siRNAs on influenza virus production in MDCK cells

		Virus production (titer in HA units)				
Exp.	siRNA	PR8 (0.001)	PR8 (0.01)	PR8 (0.1)	WSN (0.001)	WSN (0.01)
1	Mock	256	256			
	GFP-949	128	256			
	PB2-2210	16	32			
	PB2-2240	2	16			
	PB1-6	64	64			
	PB1-129	2	16			
	PB1-2257	1	4			
2	Mock	128	128			
	GFP-949	64	128			
	PA-44	64	128			
	PA-739	32	64			
	PA-2087	1	8			
	PA-2110	16	32			
	PA-2131	32	64			
3	Mock	16	64		128	
	NP-231	1	16		32	
	NP-390	4	32		64	
	NP-1496	1	1		1	
	M-37	8	32		1	
4	Mock		64	256	128	
	M-37		32	256	1	
	M-480		32	256	32	
	M-598		32	256	1	
	M-934		64	256	32	
	NS-128		32	256	64	
	NS-562		64	256	128	
	NS-589		64	256	128	
	NP-1496		1	16	1	
5	Mock		64			128
	GFP-949		64			128
	PB2-2240		8			64
	PB1-2257		8			32
	PA-2087		4			1
	NP-1496		1			1
	NP-231		8			64
	NP-390		32			128

The assays were done in the same way as in Fig. 1. Numbers in parentheses are moi values.

Inhibition of Virus Production in Embryonated Chicken Eggs. To extend the results in MDCK cells, we tested the ability of siRNAs to inhibit influenza virus production in developing chicken embryos, a widely used *in vivo* model of influenza virus infection. For siRNA transfection in the embryos, we used Oligofectamine, a lipid-based carrier that has been shown to facilitate intracellular uptake of DNA oligonucleotides (18). PR8 virus alone or virus plus siRNA was injected with Oligofectamine into the allantoic cavity of 10-day-old embryonated chicken eggs. Allantoic fluids were collected 17 h later for measurement of virus titers. When virus was injected alone (in the presence of Oligofectamine), high virus titers were detected (Fig. 2). As expected, coinjection of virus plus GFP-949 did not affect the virus titer. Coinjection of siRNAs specific for influenza virus, however, reduced virus titers. The results were concordant with those in MDCK cells. The same siRNAs (NP-1496, PA-2087, and PB1–2257) that potently inhibited influenza virus production in MDCK cells also inhibited virus production in chicken embryos, whereas siRNAs (NP-231, M-37, and PB1–129) that were less effective in MDCK cells (Fig. 1*D* and Table 1) were ineffective in chicken embryos. No significant reduction of virus titer was

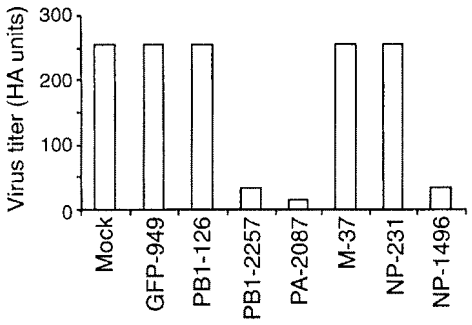

Fig. 2. siRNAs interfere with influenza virus production in embryonated chicken eggs. A mixture of siRNAs (2.5 nmol), Oligofectamine, and PR8 virus (500 pfu) was injected into the allantoic cavity of 10-day-old embryonated chicken eggs. Allantoic fluid was collected 17 h later and assayed for virus titers as in Fig. 1. Data shown are from one of two experiments.

observed when Oligofectamine was omitted. Thus, siRNAs also effectively interfere with influenza virus production in embryonated chicken eggs.

mRNA Is Probably the Direct Target of RNAi. The RNA segments present in the influenza virion are called virion RNA (vRNA, −strand, Fig. 3*A*) (1). Transcription of vRNA by the virus-encoded transcriptase produces mRNA (+ strand), which serves as a template for synthesis of viral proteins. Transcription of vRNA also produces complementary RNA (cRNA, + strand), which serves as a template for synthesizing more vRNA for new virion production. Although siRNA is known to target mRNA for degradation (10–12), siRNA may also interfere with vRNA and cRNA. This result could occur because of the complementarity of the duplex siRNA strands: the antisense strand is complementary to mRNA and cRNA, and the sense strand is complementary to vRNA. To investigate these possibilities, we used NP-1496 siRNA in which either the sense (S or +) or antisense (AS or −) strand was modified (mS:wtAS or wtS:mAS). The modification, which replaces the 2′-hydroxyl group with a 2′-*O*-methyl group in every nucleotide residue, does not affect base-pairing for duplex formation, but the modified RNA strand no longer supports RNAi (M.T.M. and P.A.S., unpublished data).

MDCK cells were electroporated with wild-type or modified NP-1496 siRNAs, followed by PR8 virus infection, and virus titer in culture supernatants was measured. High virus titers were detected in cultures with mock transfection (Fig. 3*B*). As expected, very low virus titers were detected in cultures transfected with wild-type siRNA (wtS:wtAS), and high virus titers were detected in cultures transfected with siRNA in which both strands were modified (mS:mAS). The virus titers were high in cultures transfected with siRNA in which the antisense strand was modified (wtS:mAS), whereas the virus titers were low in cultures transfected with siRNA in which the sense strand was modified (mS:wtAS). The requirement for a wild-type antisense (−) strand in the siRNA duplex to inhibit influenza virus production suggests that the target of RNAi is either mRNA (+), cRNA (+), or both.

To further distinguish between these possibilities, we examined the effect of siRNA on the accumulation of the corresponding mRNA, cRNA, and vRNA. To follow transcription in a cohort of simultaneously infected cells, siRNA-transfected MDCK cells were harvested for RNA isolation 1, 2, and 3 h after infection (before new virion release and reinfection). The viral mRNA, vRNA, and cRNA were first independently converted to cDNA by using specific primers (Fig. 3*A*). The level of each cDNA was then quantified by real-time PCR. When M-specific

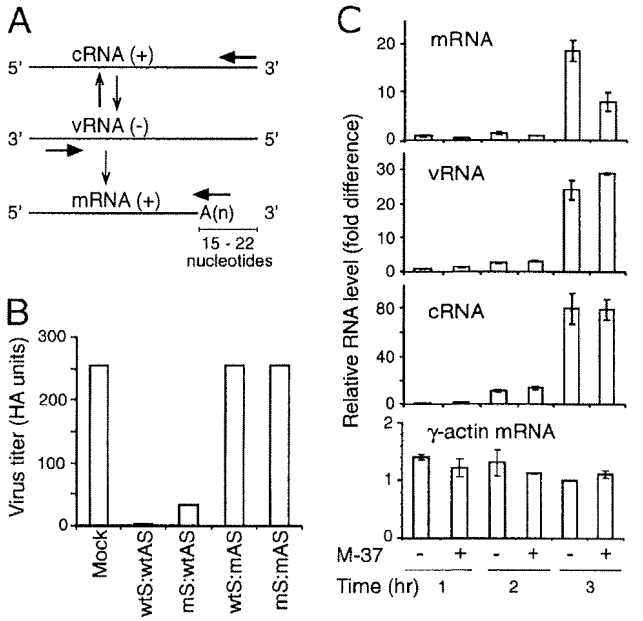

Fig. 3. mRNA is the likely target for siRNA-mediated interference. (*A*) Schematic diagram illustrating the relationship among influenza virus vRNA, mRNA, and cRNA. Whereas cRNA is the exact complement of vRNA, mRNA contains a cap structure at the 5′ end (not shown) and a poly(A) sequence that occurs at a site 15–22 nt before the 5′ end of the vRNA segment. Arrows indicate the positions of primers used to distinguish among the various RNAs during RT. (*B*) Inhibition of influenza virus production requires a wild-type (wt) antisense strand in the duplex siRNA. MDCK cells were first electroporated with siRNAs formed from wt and modified (m) strands and were infected 8 h later with PR8 virus at a moi of 0.1. Virus titers in the culture supernatants were assayed 24 h after infection as in Fig. 1. Data shown are from one of two experiments. (*C*) M-specific siRNA inhibits the accumulation of specific mRNA. MDCK cells were electroporated with M-37, infected with PR8 virus at a moi of 0.01, and harvested for RNA isolation 1, 2, and 3 h after infection. The levels of M-specific mRNA, cRNA, and vRNA were measured by RT using RNA-specific primers as indicated in *A*, followed by real-time PCR. The level of each viral RNA species was normalized to the level of γ-actin mRNA (*Bottom*) in the same sample. The relative levels of RNAs are shown as mean value ± SD. Data shown are from one of two experiments.

siRNA M-37 was used, little M-specific mRNA was detected 1 or 2 h after infection (Fig. 3*C*). Three hours after infection, M-specific mRNA was readily detected in the absence of M-37. In cells transfected with M-37, the level of M-specific mRNA was reduced by ≈50%. In contrast, the levels of M-specific vRNA and cRNA were not significantly reduced by the presence of M-37, indicating again that viral mRNA is probably the target of siRNA-mediated interference.

Some siRNAs Inhibit Accumulation of All Viral RNAs. We also examined the effect of NP-1496 siRNA on the accumulation of NP-specific mRNA, vRNA, and cRNA by RT, followed by real-time PCR. With similar results as M-specific mRNA, NP-specific mRNA was low 1 or 2 h after infection (Fig. 4*A Top*). Three hours after infection, NP mRNA was readily detected in the absence of NP-1496, whereas in the presence of NP-1496, the amount of NP mRNA remained at the background level, indicating that siRNA inhibited the accumulation of specific mRNA. However, the NP-specific vRNA and cRNA were also abolished by the presence of NP-1496 (Fig. 4*A Middle* and *Bottom*). The same results were also obtained when Northern blotting was used to detect the viral RNA in infected cells (Fig. 5). Moreover, inhibition of other viral RNAs also occurred. In the NP-1496-treated cells, the accumulation of mRNA, vRNA, and cRNA of

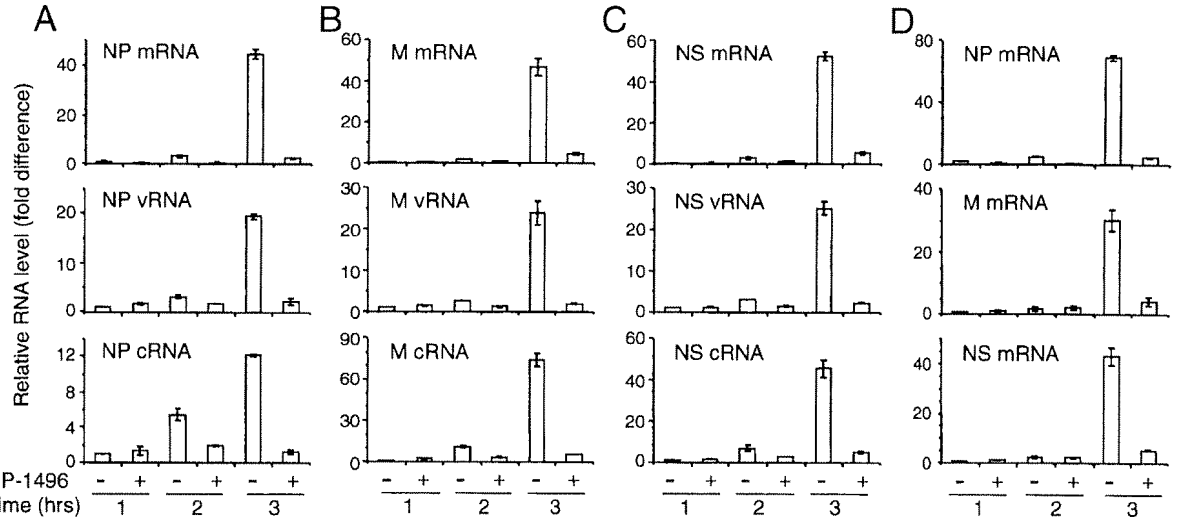

Fig. 4. NP-specific siRNA inhibits the accumulation of not only NP- but also M- and NS-specific mRNA, vRNA, and cRNA. MDCK (*A–C*) and Vero (*D*) cells were electroporated with NP-1496, infected with PR8 virus at a moi of 0.1, and harvested for RNA isolation 1, 2, and 3 h after infection. The levels of mRNA, cRNA, and vRNA specific for NP, M, and NS were measured by RT using RNA-specific primers, followed by real-time PCR. The level of each viral RNA species is normalized to the level of γ-actin mRNA (data not shown) in the same sample. The relative levels of RNAs are shown. Data shown are from one of two experiments.

M, NS, PB1, PB2, and PA genes was also inhibited (Fig. 4 *B* and *C*, and data not shown). This broad inhibitory effect was also observed for PA-2087 (data not shown). As shown above, however, M-37 inhibited the accumulation of only M-specific mRNA but not M-specific vRNA or cRNA or other viral RNAs (Fig. 3*C*). Thus, depending on their sequence and specificity, certain siRNAs exert a global effect, inhibiting accumulation of all viral RNAs.

One possible cause for the broad inhibition of viral RNA accumulation is an IFN response by the infected cells to the presence of dsRNA (17, 19, 20). Thus, we carried out the above experiments in Vero cells in which the entire IFN locus, including all α, β, and ω genes, is deleted (21, 22) (Q.G. and J.C., unpublished data). In these cells, as in MDCK cells, the accumulation of NP-, M-, and NS-specific mRNAs was inhibited by NP-1496 (Fig. 4*D*). In addition, we assayed the effect of siRNA on the levels of transcripts from cellular genes, including β-actin, γ-actin, GAPDH, and ribosomal L32. No significant difference in transcript levels of these genes was detected in the presence or absence of siRNA (Figs. 3*C* and 5 and data not shown), indicating that the inhibitory effect of siRNA is specific for viral RNAs. These results suggest that the broad inhibition of viral RNA accumulation by siRNA is not because of a cellular IFN response.

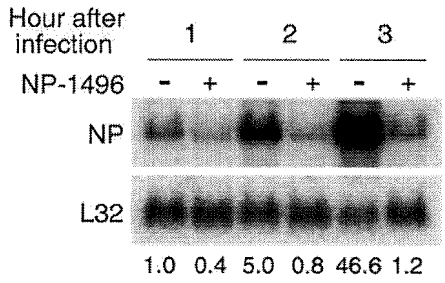

Fig. 5. NP-specific siRNA inhibits the accumulation of all NP viral RNAs. MDCK cells were electroporated with NP-1496, infected with PR8 virus at a moi of 0.1, and harvested for RNA isolation 1, 2, and 3 h after infection. Total RNA (15 μg) was separated by a 1.2% denaturing agarose gel, transferred, and hybridized sequentially with probes specific for NP (*Upper*) and the ribosomal L32 gene (*Lower*). The numbers indicate the relative levels of NP-specific RNA normalized to the levels of L32. The level of NP-specific RNA in cells that were infected for 1 h in the absence of siRNA is arbitrarily given a value of 1. The NP-specific probe was double-stranded and hybridized to mRNA, vRNA, and cRNA.

After influenza virus infection, the presence of dsRNA also activates a cellular pathway that targets RNA for degradation (17). To examine the effect of siRNA on the activation of this pathway, we assayed the levels of phosphorylated protein kinase R (PKR), the most critical component of the pathway (17). Transfection of MDCK cells with NP-1496 in the absence of virus infection did not affect the levels of activated PKR (data not shown). Infection by influenza virus resulted in an increased level of phosphorylated PKR, consistent with previous studies (19, 20, 23). However, the increase was the same in the presence or absence of NP-1496 (data not shown). Thus, the broad inhibition of viral RNA accumulation mediated by certain siRNAs is not a result of enhanced RNA degradation.

Discussion

In this report we showed that (*i*) siRNAs can potently inhibit influenza virus production, and (*ii*) some siRNAs exert their inhibitory effect by interfering with the accumulation of not only mRNA, but also other viral RNAs. These findings have significant implications for the use of siRNA for prophylaxis and therapy of influenza virus infection, and for the mechanisms underlying influenza virus transcription and replication.

Influenza virus infection is considered to have the potential to become a much more dangerous disease than at present because of easy transmission, antigenic shift and drift of the virus, and the limited efficacy of current vaccines and therapy (7). We showed that siRNAs potently inhibited influenza virus production in both cell lines and embryonated chicken eggs. Among 20 siRNAs tested, those that target NP and a component of the RNA transcriptase are especially effective, working at picomolar range and after virus infection has occurred. These results provide a basis for further development of siRNAs for prophylaxis and therapy of influenza virus infection in humans. Influenza virus naturally infects epithelial cells in the upper respiratory tract and lungs in humans. siRNAs could be conveniently administrated via intranasal or pulmonary routes. Considering that the number

of virions is probably small at the beginning of a natural infection, sufficient amounts of siRNA may be taken up by epithelial cells in the upper airways and lungs to inhibit virus replication, thus, potentially achieving preventive or therapeutic effects.

After influenza virus infection, vRNA is transcribed into both mRNA and cRNA (1). Because of the complementarity of duplex siRNA strands, siRNAs may interfere directly with mRNA and with vRNA and cRNA. We showed that viral mRNA is the direct target of siRNA-mediated interference because (*i*) the interference requires a functional antisense strand of the siRNA duplex, and (*ii*) M-specific siRNA interfered with the accumulation of M-specific mRNA, but not cRNA or vRNA. These findings are consistent with previous studies showing that siRNAs target the degradation of mRNAs that are transcribed from either cellular or viral genes (10–12, 24–26). In particular, siRNA was previously shown not to interfere with vRNA of respiratory syncytial virus (RSV) (27). As with RSV, influenza vRNA and cRNA are bound by NP, which may protect these RNAs from cleavage by RNAi machinery. It is also possible that mRNAs are targeted because they are exported to the cytoplasm, whereas vRNAs and cRNAs remain in the nucleus of influenza virus-infected cells.

We found, however, that NP- or PA-specific siRNA interfered with the accumulation of not only NP- or PA-specific mRNA, but also NP- or PA-specific cRNA and vRNA. These siRNAs also inhibited the accumulation of RNAs from other viral genes. This broad inhibition was virus-specific, as it did not significantly affect RNAs transcribed from cellular genes. This virus-specific inhibition was also observed in Vero cells, which lack the IFN α, β, and ω genes, indicating that the broad inhibition is not a result of an IFN response that shuts off all transcriptions in the infected cells. In addition, the broad inhibition was not a result of a general degradation of virus-specific RNAs because activation of protein kinase R was not affected by the presence of siRNA in infected cells. Instead, these findings reveal a critical role of newly synthesized NP and PA proteins in viral transcription and replication.

The number of NP protein molecules in infected cells has been hypothesized to regulate the levels of mRNA synthesis versus genome RNA (vRNA and cRNA) replication (1). Using a temperature-sensitive mutation in the NP protein, previous studies have shown that cRNA, but not mRNA, synthesis was temperature-sensitive both *in vitro* and *in vivo* (28, 29). NP protein was also shown to be required for elongation and antitermination of nascent cRNA and vRNA transcripts (29, 30). We found that NP-specific siRNA inhibited the accumulation of all viral RNAs in infected cells. Probably, in the presence of NP-specific siRNA, the newly transcribed NP mRNA is degraded, resulting in inhibition of NP protein synthesis. Without newly synthesized NP, further viral transcription and replication are blocked, as is new virion production.

Similarly, in the presence of PA-specific siRNA, newly transcribed PA mRNA is degraded, resulting in inhibition of PA protein synthesis. Despite the presence of 30–60 copies of RNA transcriptase per influenza virion (1), without newly synthesized RNA transcriptase, further viral transcription and replication are evidently inhibited. In contrast, the matrix (M) protein is not required until a late phase of virus infection (1). Accordingly, M-specific siRNA inhibits the accumulation of M-specific mRNA but not vRNA, cRNA, or other viral RNAs. Taken together, these findings demonstrate a critical requirement for newly synthesized NP and PA proteins in influenza viral RNA transcription and replication. The broad inhibition of all viral RNA accumulation by NP- or PA-specific siRNAs probably occurs because the RNAs are not transcribed. Both the targeted mRNA degradation and the resulting global inhibition of other viral RNA transcription make the NP- and PA-specific siRNAs especially potent inhibitors of influenza virus infection.

We thank Drs. Peter Palese and Adolfo Garcia-Sastre for kindly allowing Q.G. to visit their laboratories; Drs. Christopher Basler, Astrid Flandorfer, Luis Martinez-Sobrido, and Yurie Nakaya for teaching Q.G. various techniques for handling influenza virus; and members of the Chen and Eisen laboratories for helpful discussions. This work was supported in part by National Institutes of Health grants (AI40146, AI44478, and AI50631 to J.C.; AI44477 and CA60686 to H.N.E.; and GM34277, AI32486, and CA42063 to P.A.S.) and a Cancer Center Core Grant (to Richard Hynes). M.T.M. is partially supported by a postdoctoral fellowship from the Cancer Research Institute.

1. Lamb, R. A. & Krug, R. M. (2001) in *Fundamental Virology*, eds. Knipe D. M. & Howley, P. M. (Lippincott Williams & Wilkins, Philadelphia), pp. 725–770.
2. Fukuda, K., Bridges, C. B., Brammer, T. L., Izurieta, H. S. & Cox, N. J. (1999) *Morbid. Mortal. Wkly. Rep.* **48,** 1–28.
3. Patterson, K. D. & Pyle, G. F. (1991) *Bull. Hist. Med.* **65,** 4–21.
4. Parvin, J. D., Moscona, A., Pan, W. T., Leider, J. M. & Palese, P. (1986) *J. Virol.* **59,** 377–383.
5. Webster, R. G., Laver, W. G., Air, G. M. & Schild, G. C. (1982) *Nature* **296,** 115–121.
6. Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M. & Kawaoka, Y. (1992) *Microbiol. Rev.* **56,** 152–179.
7. Webby, R. J. & Webster, R. G. (2001) *Philos. Trans. R. Soc. London* **356,** 1817–1828.
8. Castle, S. C. (2000) *Clin. Infect. Dis.* **31,** 578–585.
9. Luscher-Mattli, M. (2000) *Arch. Virol.* **145,** 2233–2248.
10. Vaucheret, H., Beclin, C. & Fagard, M. (2001) *J. Cell Sci.* **114,** 3083–3091.
11. Sharp, P. A. (2001) *Genes Dev.* **15,** 485–490.
12. Brantl, S. (2002) *Biochim. Biophys. Acta* **1575,** 15–25.
13. Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. & Mello, C. C. (1998) *Nature* **391,** 806–811.
14. Baulcombe, D. (2002) *Curr. Biol.* **12,** R82–R84.
15. Elbashir, S., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. & Tuschl, T. (2001) *Nature* **411,** 494–498.
16. McManus, M. T. & Sharp, P. A. (2002) *Nat. Rev. Genet.* **3,** 737–747.
17. Kumar, M. & Carmichael, G. G. (1998) *Microbiol. Mol. Biol. Rev.* **62,** 1415–1434.
18. Pedroso de Lima, M. C., Simoes, S., Pires, P., Faneca, H. & Duzgunes, N. (2001) *Adv. Drug Delivery Rev.* **47,** 277–294.
19. Garcia-Sastr, A. (2002) *Microbes Infect.* **4,** 647–655.
20. Katze, M. G., He, Y. & Gale, M., Jr. (2002) *Nat. Rev. Immunol.* **2,** 675–687.
21. Diaz, M. O., Ziemin, S., Le Beau, M. M., Pitha, P., Smith, S. D., Chilcote, R. R. & Rowley, J. D. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5259–5263.
22. Diaz, M. O., Pomykala, H. M., Bohlander, S. K., Maltepe, E., Malik, K., Brownstein, B. & Olopade, O. I. (1994) *Genomics* **22,** 540–552.
23. Kim, M.-J., Latham, A. G. & Krug, R. M. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 10096–10101.
24. Jacque, J.-M., Triques, K. & Stevenson, M. (2002) *Nature* **418,** 435–438.
25. Gitlin, L., Karelsky, S. & Andino, R. (2002) *Nature* **418,** 430–434.
26. Novina, C. D., Murray, M. F., Dykxhoorn, D. M., Beresford, P., Riess, J., Lee, S.-K., Collman, R. G., Lieberman, J., Shankar, P. & Sharp, P. A. (2002) *Nat. Med.* **8,** 681–686.
27. Bitko, V. & Barik, S. (2001) *BMC Microbiol.* **1,** 34–43.
28. Medcalf, L., Poole, E., Elton, D. & Digard, P. (1999) *J. Virol.* **73,** 7349–7356.
29. Shapiro, G. I. & Krug, R. M. (1988) *J. Virol.* **62,** 2285–2290.
30. Beaton, A. R. & Krug, R. M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 6282–6286.

1: Nature. 2002 Jul 4;418(6893):38-9.

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RNA interference in adult mice.

McCaffrey AP, Meuse L, Pham TT, Conklin DS, Hannon GJ, Kay MA.

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RNA interference is an evolutionarily conserved surveillance mechanism that responds to double-stranded RNA by sequence-specific silencing of homologous genes. Here we show that transgene expression can be suppressed in adult mice by synthetic small interfering RNAs and by small-hairpin RNAs transcribed in vivo from DNA templates. We also show the therapeutic potential of this technique by demonstrating effective targeting of a sequence from hepatitis C virus by RNA interference in vivo.

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Inhibition of hepatitis B virus in mice by RNA interference.

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Hepatitis B virus (HBV) infection substantially increases the risk of chronic liver disease and hepatocellular carcinoma in humans. RNA interference (RNAi) of virus-specific genes has emerged as a potential antiviral mechanism. Here we show that RNAi can be applied to inhibit production of HBV replicative intermediates in cell culture and in immunocompetent and immunodeficient mice transfected with an HBV plasmid. Cotransfection with plasmids expressing short hairpin RNAs (shRNAs) homologous to HBV mRNAs induced an RNAi response. Northern and Southern analyses of mouse liver RNA and DNA showed substantially reduced levels of HBV RNAs and replicated HBV genomes upon RNAi treatment. Secreted HBV surface antigen (HBsAg) was reduced by 94.2% in cell culture and 84.5% in mouse serum, whereas immunohistochemical detection of HBV core antigen (HBcAg) revealed >99% reduction in stained hepatocytes upon RNAi treatment. Thus, RNAi effectively inhibited replication initiation in cultured cells and mammalian liver, showing that such an approach could be useful in the treatment of viral diseases.

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Inhibition of retroviral pathogenesis by RNA interference.

Hu WY, Myers CP, Kilzer JM, Pfaff SL, Bushman FD.

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BACKGROUND: RNA interference (RNAi) is a newly discovered cellular defense system that is known to suppress replication of genomic parasites in model organisms. It has been widely conjectured that RNAi may also serve as an antiviral system in vertebrates. RESULTS: Retroviral infection could be initiated by electroporation of cloned Rous sarcoma virus (RSV) proviral DNA into the developing chick neural tube. Coelectroporation of proviral DNA and short double-stranded RNAs matching sequences of avain retroviruses, which were designed to induce RNAi against RSV, inhibited viral replication. Replication of RSV after electroporation resulted in disruption of embryonic development and early death, but this, too, could be suppressed by RNAi against the RSV genome. RNAi could also inhibit the growth of RSV and HIV in cell culture. Analysis of the step of the retroviral life cycle that is inhibited by RNAi revealed that it primarily prevented accumulation of the viral RNAs synthesized late during infection. RNA genomes introduced in viral particles early during infection were less sensitive. CONCLUSIONS: RNAi can block retroviral infection in vertebrates. The tissue electroporation method described here should allow RNAi to be used widely to study gene function and control of infection in vertebrate animals.

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Short interfering RNA confers intracellular antiviral immunity in human cells.

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Gene silencing mediated by double-stranded RNA (dsRNA) is a sequence-specific, highly conserved mechanism in eukaryotes. In plants, it serves as an antiviral defence mechanism. Animal cells also possess this machinery but its specific function is unclear. Here we demonstrate that dsRNA can effectively protect human cells against infection by a rapidly replicating and highly cytolytic RNA virus. Pre-treatment of human and mouse cells with double-stranded, short interfering RNAs (siRNAs) to the poliovirus genome markedly reduces the titre of virus progeny and promotes clearance of the virus from most of the infected cells. The antiviral effect is sequence-specific and is not attributable to either classical antisense mechanisms or to interferon and the interferon response effectors protein kinase R (PKR) and RNaseL. Protection is the result of direct targeting of the viral genome by siRNA, as sequence analysis of escape virus (resistant to siRNAs) reveals one nucleotide substitution in the middle of the targeted sequence. Thus, siRNAs elicit specific intracellular antiviral resistance that may provide a therapeutic strategy against human viruses.

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LETTERS

RNAi-mediated gene silencing in non-human primates

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The opportunity to harness the RNA interference (RNAi) pathway to silence disease-causing genes holds great promise for the development of therapeutics directed against targets that are otherwise not addressable with current medicines[1,2]. Although there are numerous examples of *in vivo* silencing of target genes after local delivery of small interfering RNAs (siRNAs)[3–5], there remain only a few reports of RNAi-mediated silencing in response to systemic delivery of siRNA[6–8], and there are no reports of systemic efficacy in non-rodent species. Here we show that siRNAs, when delivered systemically in a liposomal formulation, can silence the disease target apolipoprotein B (ApoB) in non-human primates. *APOB*-specific siRNAs were encapsulated in stable nucleic acid lipid particles (SNALP) and administered by intravenous injection to cynomolgus monkeys at doses of 1 or 2.5 mg kg^{-1}. A single siRNA injection resulted in dose-dependent silencing of *APOB* messenger RNA expression in the liver 48 h after administration, with maximal silencing of >90%. This silencing effect occurred as a result of *APOB* mRNA cleavage at precisely the site predicted for the RNAi mechanism. Significant reductions in ApoB protein, serum cholesterol and low-density lipoprotein levels were observed as early as 24 h after treatment and lasted for 11 days at the highest siRNA dose, thus demonstrating an immediate, potent and lasting biological effect of siRNA treatment. Our findings show clinically relevant RNAi-mediated gene silencing in non-human primates, supporting RNAi therapeutics as a potential new class of drugs.

ApoB is expressed predominantly in the liver and jejunum, and is an essential protein for the assembly and secretion of very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL), which are required for the transport and metabolism of cholesterol[9]. As a large, lipid-associated protein, ApoB is not accessible to targeting with conventional therapies, but it is a highly relevant and validated disease target. Elevated ApoB and LDL levels are correlated with increased risk of coronary artery disease, and inadequate control of LDL–cholesterol after acute coronary syndromes results in increased risk of recurrent cardiac events or death[10,11]. Approaches targeting ApoB with second-generation antisense oligonucleotides have progressed to pre-clinical and clinical studies[12]. Despite progress in the management of hypercholesterolaemia using HMG-CoA reductase inhibitors and other drugs that affect dietary cholesterol, there remains a significant need for new therapeutic approaches.

We have previously demonstrated silencing of *Apob* in rodents using cholesterol-conjugated siRNAs[6]. In the current study, we used a liposomal formulation of SNALP to evaluate systemic delivery of siRNA directed towards *APOB*. Preliminary evaluations were conducted in mice. Whereas administration of the *Apob*-specific siRNA siApoB-1, without formulation or chemical conjugation, at doses higher than 50 mg kg^{-1} was previously shown to have no

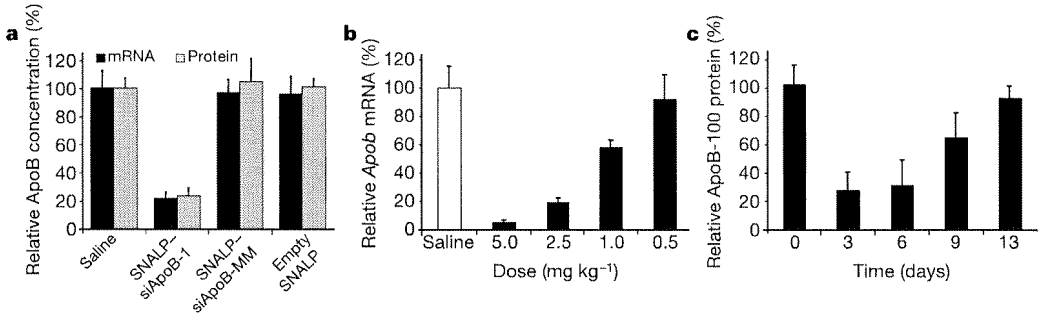

Figure 1 | SNALP-siRNA-mediated silencing of murine *Apob* is potent, specific, dose-dependent and long-lasting. **a**, Liver *Apob* mRNA levels normalized to *Gapdh* mRNA and serum ApoB-100 protein levels measured two days after single i.v. injections of saline, SNALP–siApoB-1 (1 mg kg^{-1}), mismatched SNALP–siApoB-MM (1 mg kg^{-1}) or empty SNALP vesicles (25 mg kg^{-1}) (n = 5 per group). **b**, Liver *Apob* mRNA levels normalized to *Gapdh* mRNA, assessed three days after i.v. administration of saline or 5, 2.5, 1 or 0.5 mg kg^{-1} SNALP–siApoB-2 (n = 4 per group). **c**, Serum ApoB-100 levels after i.v. administration of either saline or 2.5 mg kg^{-1} SNALP–siApoB-2 (n = 6 per group). Serum ApoB-100 levels for SNALP–siApoB-2-treated animals are relative to the saline-treated group for the same time point. Data show mean ± s.d.

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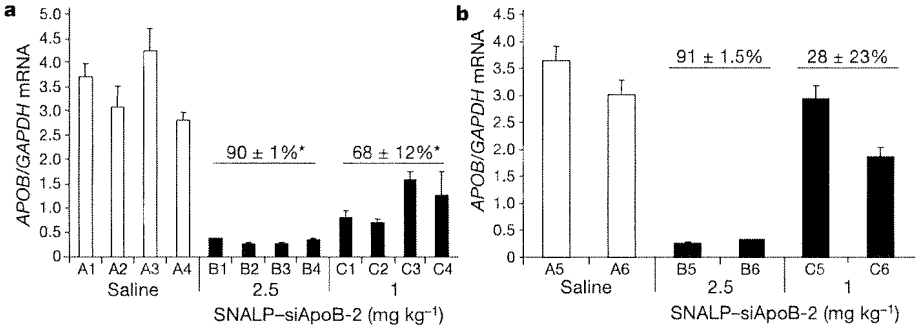

Figure 2 | Systemic silencing of *APOB* mRNA in non-human primates. **a**, **b**, Liver *APOB* mRNA levels for 12 biopsies (three isolated from each of four liver lobes) were quantified relative to *GAPDH* mRNA either 48 h (**a**, $n = 4$ animals per group) or 11 days (**b**, $n = 2$) after treatment with SNALP–siApoB-2. Data shown are mean *APOB*/*GAPDH* mRNA levels ± s.d. for each animal. Mean values (±s.d.) of the per cent *APOB* mRNA reduction relative to the saline treatment group are shown above each group. Asterisks indicate statistical significance compared with the saline-treated group ($P < 0.005$; ANOVA).

in vivo silencing activity[6], ~80% silencing of liver *Apob* mRNA and ApoB-100 protein was achieved with a single 1 mg kg^{-1} dose of SNALP-formulated siApoB-1 (Fig. 1a). In contrast, no detectable reduction was observed with a SNALP-formulated mismatched siRNA (siApoB-MM) or empty SNALP vesicles, indicating that silencing is specific to the siRNA and is not caused by the liposomal carrier. This silencing effect of SNALP-formulated siRNA represents more than a 100-fold improvement in potency compared with systemic administration of cholesterol-conjugated siApoB-1 (chol–siApoB-1) (Supplementary Fig. 1). Moreover, liposomal formulation of siRNA seems to be a general strategy for silencing hepatocyte targets, as demonstrated in mice for coagulation factor VII, green fluorescent protein and cyclophilin B (A.A., R. Constien and M.N.F., unpublished results).

As siApoB-1 was originally designed to be cross-reactive to both mouse and human ApoB genes, and we planned to conduct RNAi studies in non-human primates, a second ApoB-specific siRNA, siApoB-2, was designed to be cross-reactive with mouse, human and cynomolgus monkey ApoB genes. siApoB-2 was also selected on the basis of *in vitro* gene silencing activity and the absence of immunostimulatory activity (data not shown). Murine studies showed that encapsulated siApoB-2 showed a dose-dependent reduction in *Apob* mRNA, with >90% silencing achieved at the highest (5 mg kg^{-1}) dose (Fig. 1b). After a single 2.5 mg kg^{-1} dose of SNALP–siApoB-2, 80% silencing of liver *Apob* mRNA was associated with a 72% reduction in serum ApoB-100 protein. The silencing effect was detected for up to nine days, and was followed by recovery to normal protein levels by day 13 after treatment (Fig. 1c).

To address the therapeutic potential of this systemic RNAi approach, we evaluated the pharmacokinetics, efficacy and safety of SNALP-formulated siApoB-2 in cynomolgus monkeys. We first determined the circulating half-life of SNALP–siApoB-2 in plasma samples collected from cynomolgus monkeys ($n = 2$) receiving a single 2.5 mg kg^{-1} intravenous (i.v.) injection of the siRNA. An elimination half-life of 72 min was measured for the siRNA (Supplementary Fig. 2), compared with a 38-min half-life in mice (Supplementary Fig. 3a).

To evaluate efficacy, cynomolgus monkeys were treated with saline or SNALP-formulated siApoB-2 at doses of 1 or 2.5 mg kg^{-1} ($n = 6$ per group). siApoB-2 treatment was associated with a clear and statistically significant dose-dependent gene-silencing effect on cynomolgus liver *APOB* mRNA. Forty-eight hours after treatment, *APOB* mRNA was reduced by 68 ± 12% (mean ± s.d., $n = 4$, $P = 0.004$) and 90 ± 1% ($n = 4$, $P = 0.002$) for the 1 mg kg^{-1} and 2.5 mg kg^{-1} groups, respectively (Fig. 2a). Gene silencing was found to be consistent across the liver and correlated with detectable tissue levels of siApoB-2 (Supplementary Fig. 4). We also confirmed this *APOB* mRNA silencing to be mediated by RNAi, as demonstrated by 5′ rapid amplification of cDNA ends (RACE) analysis and identification of the predicted cleavage site, exactly ten nucleotides from the 5′ end of the antisense strand of siApoB-2 (Supplementary Fig. 5). Notably, *APOB* mRNA silencing was maintained for 11 days after the single 2.5 mg kg^{-1} treatment, with *APOB* mRNA levels still reduced by 91 ± 1.5% (Fig. 2b). Monkeys treated with the 1 mg kg^{-1} dose showed varying degrees of recovery from ApoB silencing at the day 11 time point. Although *APOB* mRNA was efficiently silenced in the liver, SNALP–siApoB-2 showed no silencing of *APOB* expressed in the jejunum (Supplementary Fig. 6), consistent with the absence of significant biodistribution of SNALP-formulated siRNAs to intestinal tissues in mice (Supplementary Fig. 3b).

The degree and persistence of RNAi-mediated silencing observed in cynomolgus monkeys far exceeds the results obtained with rodents. The lasting RNAi-mediated effects *in vivo* are consistent with observed long-lasting silencing by siRNAs in other studies[13,14], and the longer duration observed in primates may relate to species differences in the efficiency and stability of the RNA-induced

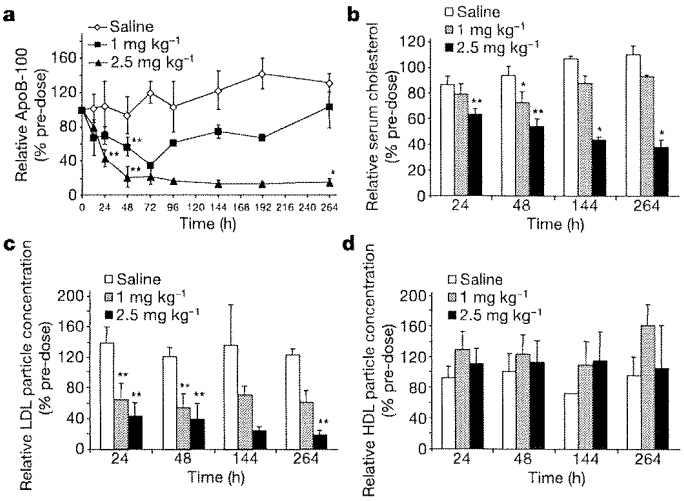

Figure 3 | Phenotypic effects of RNAi-mediated silencing of *APOB* mRNA in non-human primates. **a**–**d**, Serial plasma samples were obtained from cynomolgus monkeys treated with saline or 1 or 2.5 mg kg^{-1} SNALP–siApoB-2, and measured for ApoB-100 (**a**), total serum cholesterol (**b**), LDL (**c**) and HDL (**d**) levels. Data show levels as a percentage of pre-dose values and are expressed as mean ± s.d. Data sets collected at 0, 12, 24 and 48 h have a group size of six, and data sets collected at later time points have a group size of two. Data points marked with asterisks are statistically significant compared with saline-treated animals (*$P < 0.05$, **$P < 0.005$; ANOVA).

silencing complex (RISC), the mitotic state of hepatocytes and/or the tissue stability of the siRNA.

The expected biological effects resulting from *APOB* mRNA silencing include reduction in the blood levels of ApoB-100 protein, total cholesterol and LDL. To evaluate the kinetics of these downstream effects, we analysed plasma sampled serially from individual monkeys before and during the 11-day time course of the single-dose siApoB-2 study. Plasma ApoB-100 protein levels were reduced as early as 12 h after administration of 1 or 2.5 mg kg^{-1} SNALP–siApoB-2, reaching nadirs of 35 ± 2% and 22 ± 9% of pre-treatment levels, respectively, 72 h after treatment (Fig. 3a). Animals that received the higher siRNA dose maintained a marked reduction in ApoB protein between 2 and 11 days after treatment, consistent with the lasting effect on mRNA silencing. Monkeys that received the lower siRNA dose showed an intermediate degree of ApoB protein reduction that returned to pre-dose levels by day 11, consistent with the observed recovery in *APOB* mRNA.

Serum cholesterol levels were similarly reduced, in a dose-dependent manner and with comparable kinetics (Fig. 3b). The maximum cholesterol reduction of 62 ± 5.5% ($n = 2$, $P = 0.006$) observed for the high dose siRNA group would be considered clinically significant for patients with hypercholesterolaemia, and exceeds levels of cholesterol reduction reported clinically for currently approved cholesterol-lowering drugs.

Administration of SNALP–siApoB-2 also resulted in dramatic and rapid dose-dependent reduction in the ApoB-containing lipoprotein particle LDL. Reduction in LDL relative to pre-dose levels was observed as early as 24 h after treatment for both doses of SNALP–siApoB-2 (Fig. 3c). In contrast, there were no significant changes in circulating levels of the non-ApoB-containing high-density lipoprotein particle (HDL, Fig. 3d). The reduction in LDL persisted over the 11-day study for both siApoB-2 treatment groups, with a maximum 82 ± 7% decrease compared to pre-treatment levels observed for the high-dose group at day 11 ($n = 2$, $P = 0.003$). The time required for the biological effects to return to pre-dose levels was not determined for the high-dose group because the endpoint for this study was defined using rodent data, which indicated a faster rate of recovery. The rapid onset and lasting effect on lipoprotein metabolism suggest that siRNAs targeting *APOB* may be a valuable therapeutic strategy for achieving plaque stabilization in acute coronary syndromes[10,11], as HMG-CoA reductase inhibitors can require up to 4–6 weeks to have the desired clinical effects[15].

An important consideration for the therapeutic application of siRNA relates to its general safety, as well as to the safety profile associated with specific delivery technologies. General tolerability as well as specific toxicities (such as activation of complement, coagulation and cytokines) were evaluated for all monkeys in this study. We observed no treatment-related effects on the appearance or behaviour of animals treated with SNALP–siApoB-2 compared with saline-treated animals. There was no evidence for complement activation, delayed coagulation, pro-inflammatory cytokine production (Supplementary Table 1) or changes in haematology parameters (data not shown), toxicities that have been observed previously with treatments using related approaches[16–19]. Across a systematic evaluation, the only detected change in primates treated with SNALP–siApoB-2 was a transient increase in liver enzymes in monkeys that received the high dose of SNALP–siApoB-2. The observed transaminosis peaked 48 h after treatment and was highly variable across individual animals. These effects, which were observed only at the highest dose of SNALP–siApoB-2, were completely reversible, with normalization by day 6 notwithstanding continued biological efficacy.

Our study highlights the potential for therapeutic gene silencing using systemic RNAi in non-human primates. A single, low dose of *APOB*-specific siRNA resulted in rapid and lasting RNAi-mediated gene silencing, with associated and profound phenotypic changes. The study was limited by the premature termination of the protocol after 11 days, which prevented full evaluation of the time course for RNAi-mediated effects. Although further optimization of treatment regimen and safety profile characterization may be required, our data suggest that systemic delivery of siRNAs for targeting hepatocyte-specific genes in a higher species is possible. Furthermore, the rapid and long-lasting silencing of *APOB* using RNAi may represent a new strategy for reducing LDL–cholesterol in several relevant clinical settings.

METHODS

Additional details of the methods used are provided in the Supplementary Information.

siRNA formulation. The SNALP formulation contained the lipids 3-*N*-[(ω-methoxypoly(ethylene glycol)$_{2000}$)carbamoyl]-1,2-dimyristyloxy-propylamine (PEG-C-DMA), 1,2-dilinoleyloxy-*N*,*N*-dimethyl-3-aminopropane (DLinDMA), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and cholesterol, in a 2:40:10:48 molar per cent ratio.

***In vivo* experiments.** Saline and siRNA preparations were administered by tail vein injection under normal pressure and low volume (0.01 ml g^{-1}) for all rodent experiments. Cynomolgus monkeys ($n = 6$ per group) received either 2 ml kg^{-1} phosphate buffered saline or 1 or 2.5 mg kg^{-1} SNALP–siApoB-2 at a dose volume of 1.25 ml kg^{-1} as bolus i.v. injections via the saphenous vein. For mRNA measurements, three liver biopsies per lobe were collected 48 h ($n = 4$) or 264 h ($n = 2$) after siRNA administration.

Bioanalytical methods. The QuantiGene assay (Genospectra) was used to quantify reduction in *APOB* mRNA levels relative to the housekeeping gene *GAPDH* in lysates prepared from mouse liver or cynomolgus monkey liver and jejunum as previously described[6] but with minor variations. Mouse[6] and cynomolgus monkey ApoB-100 protein levels were quantified by enzyme-linked immunosorbent assay (ELISA). LDL and HDL lipoprotein content were determined for plasma samples (250 μl) as described previously[6].

Statistical analysis. *P*-values were calculated for comparison of SNALP–siApoB-2-treated animals with saline-treated animals using analysis of variance (ANOVA, two-factor without replication) with an alpha value of 0.05. *P*-values less than 0.05 were considered significant.

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1. Novina, C. D. & Sharp, P. A. The RNAi revolution. *Nature* 430, 161–164 (2004).
2. Shankar, P., Manjunath, N. & Lieberman, J. The prospect of silencing disease using RNA interference. *J. Am. Med. Assoc.* 293, 1367–1373 (2005).
3. Thakker, D. R. *et al.* Neurochemical and behavioral consequences of widespread gene knockdown in the adult mouse brain by using nonviral RNA interference. *Proc. Natl Acad. Sci. USA* 101, 17270–17275 (2004).
4. Bitko, V., Musiyenko, A., Shulyayeva, O. & Barik, S. Inhibition of respiratory viruses by nasally administered siRNA. *Nature Med.* 11, 50–55 (2005).
5. Palliser, D. *et al.* An siRNA-based microbicide protects mice from lethal herpes simplex virus 2 infection. *Nature* 439, 89–94 (2006).
6. Soutschek, J. *et al.* Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* 432, 173–178 (2004).
7. Song, E. *et al.* Antibody mediated *in vivo* delivery of small interfering RNAs via cell-surface receptors. *Nature Biotechnol.* 23, 709–717 (2005).
8. Morrissey, D. V. *et al.* Potent and persistent *in vivo* anti-HBV activity of chemically modified siRNAs. *Nature Biotechnol.* 23, 1002–1007 (2005).
9. Brown, M. S. & Goldstein, J. L. A receptor-mediated pathway for cholesterol homeostasis. *Science* 232, 34–47 (1986).
10. Cannon, C. P. *et al.* Intensive versus moderate lipid lowering with statins after acute coronary syndromes. *N. Engl. J. Med.* 350, 1495–1504 (2004).
11. Ridker, P. M. *et al.* C-reactive protein levels and outcomes after statin therapy. *N. Engl. J. Med.* 352, 20–28 (2005).
12. Crooke, R. M. *et al.* An apolipoprotein B antisense oligonucleotide lowers LDL cholesterol in hyperlipidemic mice without causing hepatic steatosis. *J. Lipid Res.* 46, 872–884 (2005).
13. Song, E. *et al.* Sustained small interfering RNA-mediated human immunodeficiency virus type 1 inhibition in primary macrophages. *J. Virol.* 77, 7174–7181 (2003).
14. Bartlett, D. W. & Davis, M. E. Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging. *Nucleic Acids Res.* 34, 322–333 (2006).
15. Lennernas, H. & Fager, G. Pharmacodynamics and pharmacokinetics of the HMG-CoA reductase inhibitors. Similarities and differences. *Clin. Pharmacokinet.* 32, 403–425 (1997).
16. Levin, A. A. A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. *Biochim. Biophys. Acta* 1489, 69–84 (1999).

17. Chonn, A., Cullis, P. R. & Devine, D. V. The role of surface charge in the activation of the classical and alternative pathways of complement by liposomes. *J. Immunol.* **146**, 4234–4241 (1991).
18. Hornung, V. *et al.* Sequence-specific potent induction of IFN-α by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nature Med.* **11**, 263–270 (2005).
19. Judge, A. D. *et al.* Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nature Biotechnol.* **23**, 457–462 (2005).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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INTRAVITREAL INJECTION OF VASCULAR ENDOTHELIAL GROWTH FACTOR SMALL INTERFERING RNA INHIBITS GROWTH AND LEAKAGE IN A NONHUMAN PRIMATE, LASER-INDUCED MODEL OF CHOROIDAL NEOVASCULARIZATION

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Purpose: To determine the safety and efficacy of small interfering RNA (siRNA) directed against vascular endothelial growth factor (VEGF) in a nonhuman primate model of laser-induced choroidal neovascularization (CNV).

Methods: Each animal received laser rupture of Bruch's membrane to induce CNV in both eyes. Each animal was then randomized to receive 0.05 mL of either vehicle alone or VEGF siRNA at 70 μg, 150 μg, or 350 μg in both eyes by intravitreal injection. Eyes were monitored weekly by ophthalmic examination, color photography, and fluorescein angiography for 36 days after laser injury. Electroretinograms were measured at baseline and at 5 weeks after laser. CNV on fluorescein angiograms were measured for area and graded for clinically significant leakage in a standardized, randomized, and double-masked fashion on days 15, 22, 29, and 36 after laser.

Results: VEGF siRNA did not cause any change in electroretinographic, hemorrhage, inflammation, or clinical signs of toxicity. A single administration of VEGF siRNA significantly inhibited growth of CNV and attenuated angiographic leakage in a dose-dependent manner.

Conclusion: Intravitreal injection of VEGF siRNA is capable of inhibiting the growth and vascular permeability of laser-induced CNV in a nonhuman primate in a dose-dependent manner. This study demonstrates preclinical proof of a principle that supports proceeding to clinical studies of VEGF siRNA in patients with exudative age-related macular degeneration.

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Age-related macular degeneration (AMD) is the leading cause of visual loss in the developed world and is characterized by the development of choroidal neovascularization (CNV).[1] Approved treatments such as macular photocoagulation and photodynamic therapy are limited to a subpopulation of patients and have limited efficacy.[2,3] Advances in the understanding of the pathophysiology of AMD have led to the study of novel pharmacologic treatments. In particular, several of these treatments have targeted vascular endothelial growth factor (VEGF) by binding and inactivating this protein.[4–6] However, the enclosed nature of the eye and the need to deliver a molar excess to block the effects of VEGF protein limit the dosing of these molecules based on toxicity and deliverability rather than maximum efficacy.[5,6]

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SW and SJR are employees of Acuity Pharmaceuticals. MJT and AJB are consultants for Acuity Pharmaceuticals. MJT and SJR are cofounders of Acuity Pharmaceuticals and hold substantial equity.

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Recently, a novel method of post-transcriptional silencing of gene expression, called RNA interference (RNAi), was discovered.[7] RNAi is a conserved cellular mechanism that silences the expression of a protein in a specific and potent fashion by utilizing double-stranded RNA (dsRNA) molecules that target a particular messenger RNA (mRNA). Unlike single-stranded antisense RNA, which is designed to directly bind and inactivate a single corresponding mRNA, dsRNA binds a complex of proteins that uses the dsRNA sequence to seek and destroy homologous mRNA in a multiple turnover kinetic fashion. By activating the RNAi mechanism, one dsRNA can destroy hundreds of targeted mRNA, which will result in the silencing of potentially thousands of protein molecules.[8] Because of the potential of one molecule to suppress the expression of hundreds to thousands of protein molecules, RNAi is an ideal therapeutic strategy for the eye, which, because of its limited volume, requires potent molecules for effective local administration. Furthermore, because mRNA stabilization rather than transcription accounts for a significant proportion of VEGF upregulation,[9] post-transcriptional gene silencing by RNAi would result in a prolonged downregulation of VEGF production.

Although differing lengths of dsRNA can mediate RNAi, long dsRNA is processed by the cell to produce small interfering RNA (siRNA), a 21-nucleotide length dsRNA that acts as a true intermediate for the process of RNAi.[10] We have shown previously that siRNA designed against VEGF mRNA can silence gene expression and inhibit the development of laser-induced CNV in the mouse eye.[11] In this study, we developed a single, intravitreally delivered siRNA (Cand5) that potently targets human VEGF and has been confirmed both by sequence homology and in cell-based assays to target monkey VEGF. We used this siRNA to preclinically test its ability to inhibit laser-induced CNV in the nonhuman primate.

The laser-induced CNV model in a nonhuman primate was used to test both currently approved and investigational treatments for AMD.[12,13] The purpose of this study is to determine the toxicity of an intravitreal injection of siRNA against VEGF and to evaluate its ability to attenuate growth and leakage of laser-induced CNV in the nonhuman primate.

The RNAi mediated against VEGF is a potent strategy for inhibiting growth and leakage of CNV in macular degeneration. We demonstrate that at concentrations far below the maximum deliverable dose, one administration of VEGF siRNA can significantly inhibit both growth of CNV and VEGF-induced vascular permeability in a dose-dependent manner and with prolonged duration.

Materials and Methods

Animals

Eight adult cynomolgus monkeys (*Macaca fascicularis*), obtained from Sierra Biomedical, Reno, Nevada, were used in accordance with the guidelines of the Association of Research in Vision and Ophthalmology on the use of animals in research and according to the animal care guidelines of Sierra Biomedical. Before we performed surgical procedures, examination procedures, and electroretinography, the animals were anesthetized with intramuscular ketamine HCl at a dose of 10 mg/kg, followed with intravenous administration of ketamine and diazepam at a dose of 0.5 mg/kg. Before fundus examination or photography, the animals' eyes were dilated with 1% tropicamide.

Induction of experimental choroidal neovascularization

The CNV membranes were induced in the macular region. Twelve symmetrical spots were produced using 75 μm spot size, 0.1 second duration, and power settings ranging from 450 mW to 550 mW. To control for varying choroidal pigmentation, test spots in the peripheral retina were performed to calibrate the power required to produce a break in Bruch's membrane, indicated by the formation of a vapor bubble and small hemorrhage. Once power settings had been determined, 12 spots were placed between the vascular arcades in a symmetrical fashion. Care was taken to avoid lasering the fovea. During laser application, each spot was graded for the presence of a vapor bubble and accompanying small hemorrhage, which signified a break in Bruch's membrane. Fundus photographs were taken immediately after laser was performed. Spots that did not break Bruch's membrane were recorded and excluded from analysis. In total, only two spots were excluded.

Intravitreal injection

A siRNA molecule directed against VEGF, referred to as Cand5, was provided by Acuity Pharmaceuticals (Philadelphia, Pennsylvania). Cand5 was produced as described elsewhere.[11] Immediately after laser, eight monkeys were randomized to receive 0.05 mL of either vehicle or one of three doses of Cand5 (70 μg, 180 μg, 350 μg), which was injected into the midvitreous cavity using a 1 mL tuberculin syringe and a 30-gauge needle. The eyes were entered 2 mm posterior to the limbus. Injections were monitored for evidence of reflux or inadequate injection. If injection resulted in extensive reflux of substance, this was

recorded and eyes that had failed injections were excluded from analysis. Two eyes were excluded due to inadequate injections.

Ophthalmoscopic examination

Indirect ophthalmoscopy and slit-lamp biomicroscopy were performed before injection and weekly thereafter. Each eye was scored for evidence of inflammation using a standard inflammation grading system.[14] Slit-lamp biomicroscopy was performed to evaluate cataract formation, and the posterior pole was also examined for evidence of retinal detachment.

Electroretinogram

Electroretinograms were recorded before study and 5 weeks after laser induction. Electroretinograms were obtained with an Epic 2000 (LKC technologies, Gaithersburg, Maryland). Before electroretinography, animals were dark-adapted by being placed in a dark room for 30 minutes. After dark-adaptation, the animals were sedated and their eyes dilated. A contact lens and electrodes were placed. Calibration of the Epic 2000 was performed before testing and after testing. Photopic, scotopic, and flicker measurements were obtained using standardized light flash regimens.

Fluorescein angiography and fundus photography

Color photography and fluorescein angiography were performed with a digital fundus camera connected to the Imagenet system (Topcon TRC-50EX; Topcon, Paramus, New Jersey). Standardized photographs and fluorescein angiograms were taken for each animal at baseline and on days 15, 22, 29, and 36 after laser induction. Fluorescein angiography was performed by injecting fluorescein dye (0.1 mL/kg of 10% fluorescein sodium) intravenously and taking multiple pictures 3 seconds to 300 seconds after injection of the dye.

Fluorescein angiographic spot size measurement

The area of CNV was measured from representative early frame angiograms for each eye at each timepoint. Early frame angiograms were selected to avoid leakage seen on late frames. The representative images were selected for gradeability and represented images that were in or immediately after arteriovenous phase. The images were randomized and masked so that the reviewer was double-masked as to the animal, the time the photograph was taken, and the treatment. Using NIH Image software (Scion Corp., Frederick, Maryland), area measurements were made for each spot by tracing the neovascular area and recording the area measurement. The area measurements were performed on all lesions using a fixed calibration. To normalize the data, the area of the laser spot was subtracted from the measurement to represent the growth of CNV greater than the laser spot. Each digital image was unaltered.

Fluorescein angiogram grading

Representative early and late frame angiograms were selected from each eye for each timepoint. Early frames were selected for gradeablity and timing that demonstrated arteriovenous phase or immediately after arteriovenous phase. The range of times was from 3 seconds to 45 seconds. Late-phase angiograms were selected for gradeability and timing that was greater than 280 seconds. These images were randomized and double-masked so that the reviewer was masked to the animal, the time the photograph was taken, the time frame of the angiogram, and the treatment.

Each image was analyzed by an experienced angiogram reviewer (AJB). Each angiogram was graded for the presence or absence of CNV, presence or absence of coalescence, and presence or absence of hemorrhage. Each laser spot was graded for degree of leakage on a standardized scale of 0 to 5. Grading scores were defined as follows: 0, no hyperfluorescence; 1, mild speckled hyperfluorescent staining; 2, moderate hyperfluorescent staining; 3, mild lesion leakage; 4, moderate lesion leakage; 5, extensive lesion leakage. Clinically significant fluorescein leakage was assigned a grade of 4 or higher.

Histopathology

After the fifth-week fluorescein angiogram and electroretinogram, animals were killed under deep anesthesia and the eyes were carefully enucleated and placed in 10% formalin overnight. The eyes were then grossly dissected and the nasal aspect of the globe removed. The globe was then processed and embedded in paraffin, with care taken to orient the eye so that the area of interest (macular region) could be sectioned. Serial sections were performed and stained with hematoxylin and eosin. Slides were observed and photographed under brightfield illumination microscopy (Leica, Wetzlar, Germany) Sections were correlated with fluorescein angiograms.

Statistical analysis

For the CNV area analysis, because we are interested in the growth of CNV beyond the laser spot, we normalized all the data points to the size of the original laser spot by subtracting the area of the original

laser spot (0.24) from each CNV area measurement. The normalized CNV area measurements were treated as continuous variables and analyzed by generalized equation estimation (GEE)[15] to account for the correlations from the repeated measurements from laser spots over time from the same eye. Percentage of fluorescein leakage of grade 4 or more was determined as evidence of leakage, and the differences among Cand5 dose levels were also compared by performing GEE. All the data analysis was performed using SAS 8.2 (SAS, Cary, North Carolina).

Results

Inflammation and electroretinography

Throughout the experiment, no inflammation, cataract formation, retinal detachment, or vitreous hemorrhage was noted in any of the animals. Electroretinograms obtained at baseline and during the fifth week after laser showed no significant change between the baseline and the post-injection timepoint in any of the treated or control animals.

Area measurements

To determine the overall effect of one injection of siRNA on inhibition of laser-induced CNV, the areas of CNV for all timepoints were combined and averaged. This analysis demonstrated that all three doses of Cand5 inhibited growth of neovascular area as compared with findings in control animals ($P < 0.0001$ for 70 μg, 180 μg, and 350 μg compared with control). Although the 350 μg dose of Cand5 demonstrates a larger effect on decreasing neovascular growth than the other two dose levels, the differences are not statistically significant ($P > 0.05$). Overall, the area of CNV was reduced by more than 50% of the normalized control area when Cand5 was injected after laser injury.

To assess the efficacy of Cand5 over time, we measured growth of CNV area beyond the laser spot at days 15, 22, 29, and 36 after laser injury and intravitreal injection. At all doses and at each timepoint, the area of neovascularization was significantly lower in the treated eyes than in control eyes ($P < 0.001$ for each timepoint compared with control) (Figure 1). Thus, with one injection of Cand5, neovascular growth remains inhibited throughout the 36-day follow-up period.

Vascular permeability

In addition to size, a single injection of Cand5 was capable of decreasing leakage. Both early and late

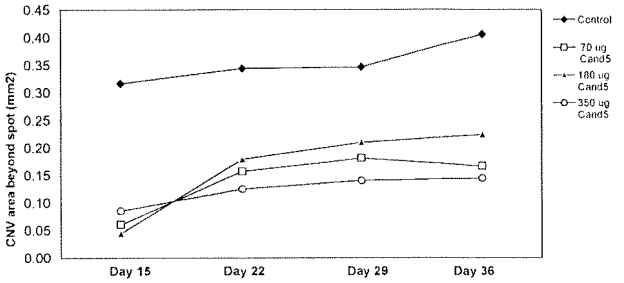

Fig. 1. Growth of choroidal neovascularization beyond the laser spot was measured from fluorescein angiograms at days 15, 22, 29, and 36 after laser induction in eyes injected with control (PBS) or one of three doses of Cand5 (siRNA targeted against VEGF). There was a significant difference between all treatment groups at each timepoint compared with control values ($P < 0.001$ for all timepoints).

fluorescein angiograms taken on day 36 demonstrate that leakage was inhibited in Cand5-treated eyes. The eye that received injection of vehicle demonstrated a large confluent bridging lesion (Figure 2A) and extensive late leakage (Figure 2B). In contrast, an eye treated with 350 μg of Cand5 demonstrated an imperceptible increase in size to the laser lesion (Figure 2C) and no late leakage of any of the laser spots (Figure 2D).

Through analysis of angiograms at each dose level, the percentage of spots that had clinically significant leakage as defined by a grade of 4 or more could be calculated for each timepoint (Figure 3). The control eyes had as much as 70% (33/47) of spots with active leakage, as compared with 27% (13/48), 6% (3/48), and 4% (1/24) of spots in eyes injected with 70 μg, 180 μg, and 350 μg, respectively, of Cand5 at Day 15; this shows a significant dose-response effect ($P < 0.0001$). Although the percentage of spots with leakage decrease with time ($P = 0.02$), the difference between treated and control eyes are still significant ($P = 0.0007$). The dose response for clinically significant leakage persists at every timepoint and can be represented as a classic pharmacologic dose-response curve (Figure 4).

To confirm that angiographically measured and graded lesions represented CNV, angiograms were correlated to histopathologic findings. Histopathology confirmed that angiographically measured CNV that stained or leaked represented subretinal neovascularization and that the extent of angiographically measured lesions corresponded to the histologic edge of the lesions (Figure 5).

Discussion

Visual loss from exudative macular degeneration results from both vascular permeability and CNV. VEGF, which is both an endothelial growth factor and

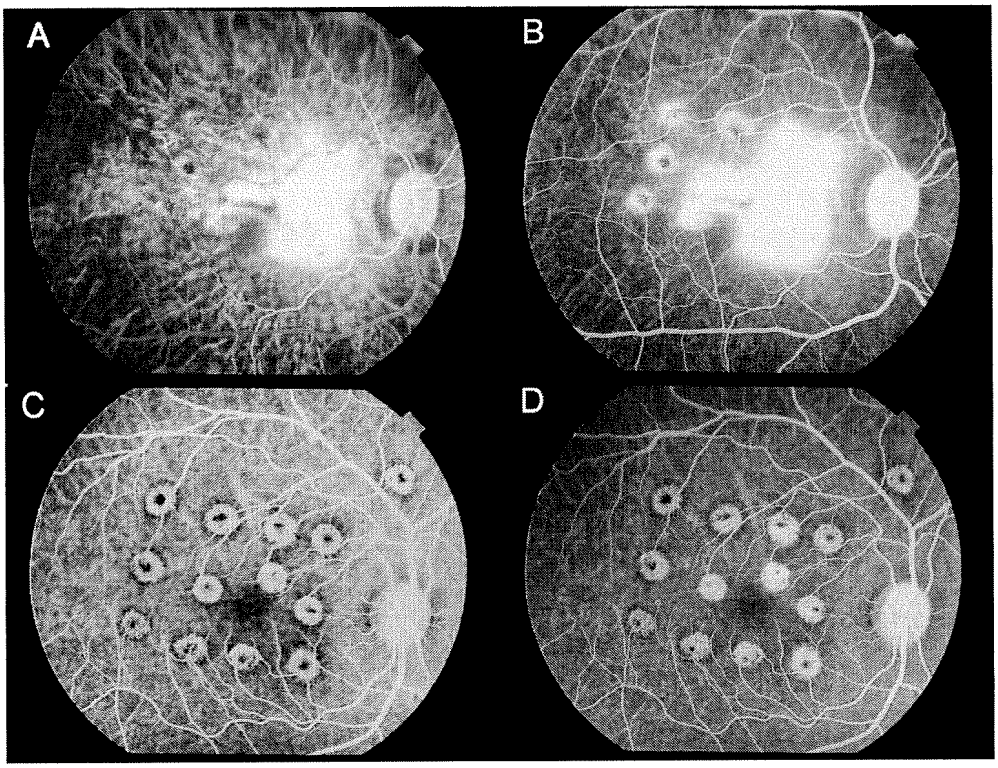

Fig. 2. Fluorescein angiograms taken 36 days after laser injury and intravitreal injection of either the control agent or Cand5. Early angiogram (during arteriovenous phase, 3 seconds) (**A**) and late angiogram (302 seconds) (**B**) of animal injected with the control agent. Early (immediately after arteriovenous phase, 30 seconds) (**C**) and late angiogram (296 seconds) (**D**) of animal injected with 350 μg of Cand5. The control animal has both large choroidal neovascularization and extensive leakage in early and late frames. The animal receiving the Cand5 shows choroidal neovascularization that does not extend past the laser spot and no leakage on late frames.

a permeability factor, plays a central role in the development of exudative AMD. By inhibiting VEGF action, both permeability and angiogenesis can be targeted. Recent clinical trials have shown that attenuating vascular permeability by the inhibition of VEGF can result in visual improvement.[6]

There are currently two inhibitors of VEGF, an aptamer and an antibody fragment, that are under commercial development for the treatment of exudative AMD. These two molecules both require direct binding of the VEGF protein on a molar-to-molar ratio to neutralize VEGF. Although both molecules are capable of inhibiting ocular neovascularization in animal models, frequent administration is required to maintain a therapeutic level of VEGF inhibition.[4,5,16] Furthermore, the requirement for frequent administration has been carried forward to the clinical trials,

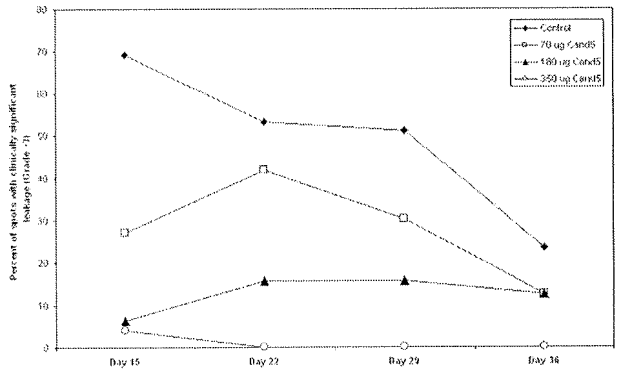

Fig. 3. Neovascular spots on angiograms were graded on a scale of 0 to 5 at days 15, 22, 29, and 36 after laser induction in eyes injected with the control agent or one of three doses of Cand5. Spots with evidence of neovascular leakage was assigned a grade of 4 or above, with the higher grade representing greater amount of leakage. At each timepoint, the percentage of neovascular spots with no significant leakage (Grade 3 or less) was higher for all three Cand5 doses as compared with control. Furthermore, increasing doses of Cand5 were associated with increasingly higher percentages of spots with no leakage.

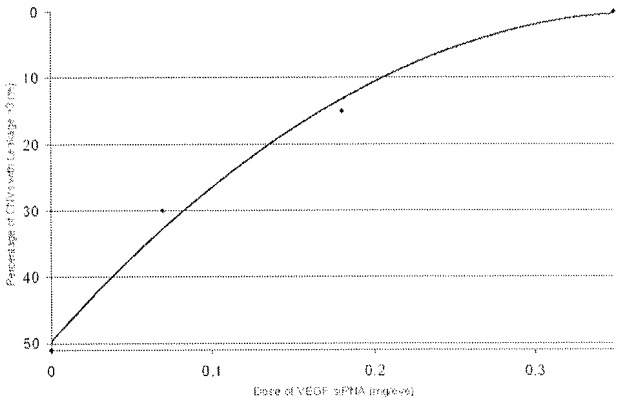

Fig. 4. The percentage of neovascular spots with no clinically significant leakage plotted against dose of Cand5 on day 29 follows a dose-response curve.

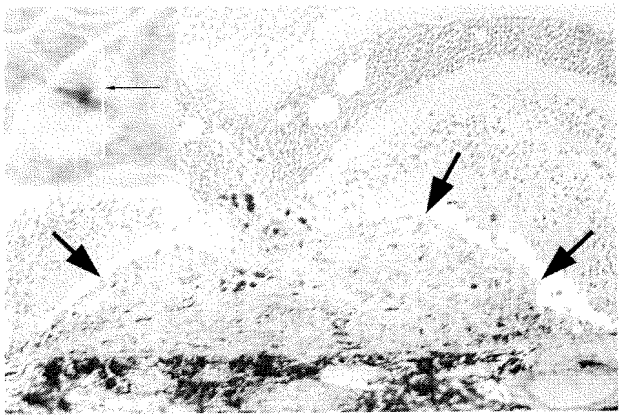

Fig. 5. Histopathologic cross section of laser-induced choroidal neovascularization that was graded with a leakage score of 3. The thin arrow and line on the angiogram shows the area ram that correlates with the tissue section. The section is stained with hematoxylin and eosin and is photographed under a ×10 microscope objective. Arrowheads point to the edge of the subretinal neovascular membrane.

which require patients to be injected every 4 to 6 weeks.

Although the advancement of these VEGF antagonists into clinical trials have validated VEGF as a therapeutic target for the treatment of AMD, the limitations of these molecules dictate the dosing and frequency of treatment. These limitations provide an opportunity to develop better VEGF inhibitors that can potentially be administered less frequently and act more potently. RNAi mediated by a siRNA against VEGF may represent this more potent and longer lasting therapeutic agent. On a molar-to-molar basis, a siRNA against VEGF is conceivably 100 to 1000 times more potent than VEGF protein antagonists. In contrast to protein antagonism, the prevention of VEGF production could potentially translate into a much longer interval between administrations, in addition to the potential for greater efficacy and decreased toxicity.

In this study, we sought to demonstrate the efficacy of RNAi in a clinically relevant model of CNV. Because of the limitations of this model and its propensity to regress spontaneously, it is not possible to directly test the full duration of effect. What we have shown in this study is that a single intravitreal injection of siRNA targeting VEGF can prevent growth of CNV and attenuate vascular leakage of CNV in a dose-dependent fashion for at least 5 weeks.

The VEGF-neutralizing fragment of monoclonal antibody against VEGF (Lucentis, Genentech, South San Francisco, California) is injected every 4 weeks and is currently under clinical investigation. This molecule was also tested in the laser-induced model of CNV in a nonhuman primate. To obtain results similar to the inhibition of leakage seen in our study, the animals required injections of rhuFAB VEGF every 2 weeks and obtained inhibition of leakage similar to our findings with a single administration of our drug.[16] With the neutralizing antibody fragment, however, the experimental eyes developed varying degrees of inflammation with their first injection and subsequent injections. In our study, we noted no inflammation, and a single injection as compared with weekly injections resulted in similar inhibition of vascular permeability, especially at our higher dose. Growth of CNV was not analyzed in the Lucentis study.

A single injection of our high dose suppressed leakage throughout the follow-up period, indicating that the duration of action on vascular permeability with one injection of Cand5 is at least 5 weeks, especially at our higher dose. Furthermore, because the maximum solubility of Cand5 in an aqueous medium is greater than 200 mg/mL (unpublished data), we are capable of dosing at much higher levels. At the dose levels used in this study, no evidence of inflammation or toxicity was noted. Formal animal toxicity studies are necessary to determine the highest acceptable dose level that can be delivered to the eye.

Although formal toxicity studies are necessary, our preliminary toxicity evaluation, which included ophthalmic examinations, histologic examinations, and electroretinograms, suggests that injections of siRNA into the eye are safe. A pegylated RNA molecule, Macugen (Eyetech Pharmaceuticals, New York, New York), which is delivered intravitreally every 6 weeks has shown efficacy in clinical trials for macular degeneration and demonstrates an excellent safety profile.[5,6] Unlike a pegylated aptamer, siRNA is a much smaller molecule, is not modified, is many-fold more soluble, and mediates a 100-fold more potent mechanism of VEGF suppression, RNA interference.

The ability of siRNA directed against VEGF to significantly inhibit choroidal neovascular growth and leakage is important for several reasons. This study illustrates the potency of siRNA as a therapeutic class of molecule. As a therapeutic agent, siRNA is potent, is highly soluble, shows no toxicity, and appears to have a longer duration of action then other available therapeutic molecules. This study also represents, to our knowledge, the first in vivo demonstrations of siRNA-mediated RNAi in a nonhuman primate administered using a clinically feasible route of administration.

In summary, this successful prevention of choroidal neovascular lesion growth and leakage in a nonhuman primate model following intravitreal administration of Cand5 is the first demonstration of therapeutic RNA interference in a primate model of disease. In addition,

this preclinical proof of principle provides a guideline for dosing in humans and provides strong support for advancing this molecule into clinical trials for the treatment of wet age-related macular degeneration.

Key words: age-related macular degeneration, choroidal neovascularization, dsRNA, nonhuman primate, RNAi, RNA interference, siRNA, small interfering RNA, vascular endothelial growth factor

References

1. Hyman L. Epidemiology of eye disease in the elderly. Eye 1987;1:330–341.
2. Macular Photocoagulation Study Group. Subfoveal neovascular lesions in age-related macular degeneration: Guidelines for evaluation and treatment in the macular photocoagulation study. Arch Ophthalmol 1991;109:1242–1257.
3. Verteporfin Roundtable 2000 and 2001 Participants; Treatment of Age-Related Macular Degeneration with Photodynamic Therapy (TAP) Study Group Principal Investigators; Verteporfin in Photodynamic Therapy (VIP) Study Group Principal Investigators. Guidelines for using verteporfin (Visudyne) in photodynamic therapy to treat choroidal neovascularization due to age-related macular degeneration and other causes. Retina 2002;22:6–18.
4. Adamis AP, Shima DT, Tolentino MJ, et al. Inhibition of vascular endothelial growth factor prevents retinal ischemia-associated iris neovascularization in a nonhuman primate. Arch Ophthalmol 1996;114:66–71.
5. Eyetech Study Group. Preclinical and Phase 1A clinical evaluation of an anti-VEGF pegylated aptamer (EYE001) for the treatment of exudative age-related macular degeneration. Retina 2002;22:143–152.
6. Eyetech Study Group. Anti-vascular endothelial growth factor therapy for subfoveal choroidal neovascularization secondary to age-related macular degeneration: Phase II study results. Ophthalmology 2003;110:979–986.
7. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 1998;391:806–811.
8. Hannon GJ. RNA interference. Nature 2002;418:244–251.
9. Shima DT, Deutsch U, D'Amore PA. Hypoxic induction of vascular endothelial growth factor (VEGF) in human epithelial cells is mediated by increases in mRNA stability. FEBS Lett 1995;370:203–208.
10. Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev 2001;15:188–200.
11. Reich SJ, Fosnot J, Kuroki A, et al. Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. Mol Vis 2003;9:210–216.
12. Husain D, Kramer M, Kenny AG, et al. Effects of photodynamic therapy using verteporfin on experimental choroidal neovascularization and normal retina and choroid up to 7 weeks after treatment. Invest Ophthalmol Vis Sci 1999;40:2322–2331.
13. Ciulla TA, Criswell MH, Danis RP, Hill TE. Intravitreal triamcinolone acetonide inhibits choroidal neovascularization in a laser-treated rat model. Arch Ophthalmol 2001;119:399–404.
14. American Academy of Ophthalmology. Basic and Clinical Science Complete Course. 9 vols. San Francisco: American Academy of Ophthalmology, 1995.
15. Zeger SL, Liang KY, Albert PS. Models for longitudinal data: A generalized estimating equation approach. Biometrics 1988;44:1049–1060.
16. Krzystolik MG, Afshari MA, Adamis AP, et al. Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment. Arch Ophthalmol 2002;120:338–346.